Cuticle degrading proteases of Metarhizium anisopliae enzyme regulation and gene cloning

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CUTICLE-DEGRADING PROTEASES OF *METARHIZIUM ANISOPLIAE*: ENZYME REGULATION AND GENE CLONING

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Dedicated, with love to

Joy, Emma and Glen

CONTENTS

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ABSTRACT

The regulation of two cuticle-degrading proteases, a chymoelastase (Pr1) and a trypsin (Pr2) from the entomopathogenic fungus Metarhizium anisopliae has been studied.

The pathogenic isolate ME1 when grown in buffered liquid cultures containing ground insect cuticle as sole carbon and nitrogen source, produced higher levels of Pr1 than in cultures containing other polymers including the insoluble proteins elastin and collagen. Correlating fungal growth (by estimation of ergosterol content) and Pr1 production in these cultures demonstrated that Pr1 was specifically induced by a component of insect cuticle. Peptides released by the hydrolysis of insect cuticle by pure preparations of Pr1 or Pr2 were capable of inducing Pr1 when added to cultures at non-repressing levels. Pr1 was not induced by deproteinated cuticle or other cuticular components (cuticular lipids or chitin) suggesting that Pr1 was induced by a component of cuticular protein. This is the first demonstration of the specific induction of a microbial protease.

The production of Pr2 was enhanced when the fungus was grown in cultures containing a range of both soluble and insoluble proteins as sole source of carbon and nitrogen. Analysis of mycelial dry weight in cultures containing the soluble protein BSA indicated that Pr2, in common with other fungal proteases, is induced by a number of unrelated proteins.

The purification and partial sequencing of these two proteases enabled the construction of gene specific oligonucleotides which were used to screen a genomic library, constructed in the lambda replacement vector EMBL3. Putative positive clones, hybridising at high stringency were identified.

A cDNA fragment of the Pr1 gene was amplified using RACE PCR. Partial sequencing of the fragment identified known peptide sequences in one of the open reading frames, thus confirming that the amplified fragment was part of the Pr1 gene.

The role of cuticle-degrading proteases during infection and the potential use of genetic engineering for studying the fungal pathogenesis of insects and in strain improvement is discussed.

ABBREVIATIONS

ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
Bz	benzoyl
C	carbon
ca.	circa
CM	complete medium
d	day
Da	daltons
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylene diamine tetraacetic acid
g	gram
HPLC	high performance liquid chromatography
hr	hour
Kb	kilobases
M	Moles or Molar
min.	minute
N	nitrogen
NA	nitroanilide
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pfu	plaque forming units
pI	iso-electric point
RH	relative humidity
RNA	ribonucleic acid

RNase	ribonuclease
SDA	Sabouraud dextrose agar
SDS	sodium dodecyl sulphate
Suc	succinyl
UV	ultraviolet
vol.	volume
wt.	weight

1. INTRODUCTION

Deuteromycete entomopathogenic fungi, in particular Metarhizium anisopliae, have great potential for use in pest control. Entomogenous fungi usually infect the host by penetrating the host cuticle by a combination of mechanical pressure and enzymatic degradation (Charnley, 1984). The cuticle-degrading enzymes of M. anisopliae have been well studied, in particular by St. Leger and co-workers (reviewed by Charnley and St. Leger, 1991). Such biochemical studies have provided strong evidence to suggest the cuticle-degrading protease Pr1 (a chymoelastase) as a pathogenicity and/or virulence determinant. Another protease, Pr2 (a trypsin) has also been characterised, but appears to play a minor role in infection. However, to date no primary determinants of pathogenicity have been unequivocally proven.

The main objectives of the work reported below were to clone the genes that encode Pr1 and Pr2 and to study in detail the regulation of these enzymes. Applying some of the molecular genetic techniques developed for other filamentous fungi to M. anisopliae should facilitate a better understanding of the role of these proteases in pathogenesis. A knowledge of the mechanisms involved in the infection process and the conclusive identification of determinants of pathogenicity and/or virulence could enable the engineering of more virulent strains of entomopathogenic fungi and therefore enhance the possibility of such fungi being used in pest control programmes.

1.1. LITERATURE REVIEW

1.1.1. Microbes as biocontrol agents.

It has been known for many years that certain species of bacteria, viruses and fungi can cause natural infections of insects and many of these entomopathogens have potential as commercial pest control agents. Increased public awareness of environmental issues and problems of insect resistance to chemical insecticides has led to a renewed interest in the use of biological control agents. However, at present, biological control agents form less than 1% of the total pesticide sales (Jutsum, 1988), the majority of which are products relating to the bacterium Bacillus thuringiensis (see Feitelson et al., 1992).

Insect pathogenic bacteria belonging to a number of genera including Clostridium, Pseudomonas and Serratia have been reported. Most have not been developed commercially due to inconsistent virulence, difficulty in culture or problems relating to safety (Luthy, 1986). Three species of gram-positive, spore forming Bacillus, namely, B. thuringiensis, B. sphaericus and B. popilliae have potential for insect control.

Highly specific insecticidal toxins are produced by B. thuringiensis as crystalline inclusions during sporulation. Most insecticidal strains of B. thuringiensis are active against

larvae of certain Lepidoptera, but some show some specificity towards species of Diptera and Coleoptera. The toxins are products of single genes, several of which have been cloned and sequenced (Hofte and Whiteley, 1989). The crystalline inclusions dissolve at alkaline pH in the insect gut, releasing a protoxin, which is in general, activated by gut proteases. The toxins appear to act by disturbing the osmotic balance in cells of the gut epithelium causing them to swell and lyse, resulting in the eventual death of the larvae (Ellar, 1990). Despite the existence of many B. thuringiensis strains few varieties are commercially available (see Payne, 1988). Strain kurstaki HD1 which is devoid of β-exotoxin (an adenine nucleotide with some vertebrate toxicity) has been most extenively used, as it has a relatively broad spectrum against lepidopteran larvae (Luthy, 1986). B thuringiensis var. israelensis (Luthy, 1986) and B. sphaericus (Berry, 1990) are showing promise for the control of the biting blackflies and mosquitoes, both important vectors for human disease. Strain improvement of B. thuringiensis is dominated by genetic engineering (Payne, 1988), with efforts targeted at improving host range and increasing toxicity and environmental persistence. B. thuringiensis toxin genes have been cloned into a variety of crop plants with the view to producing pest resistant cultivars (see Brunke and Meeusen, 1991).

Viruses with a range of morphological and biochemical properties have been isolated from diseased insects (see Payne, 1982) although frequently many of these viruses fail to induce lethal

infections. Members of the Baculoviride family of viruses frequently produce lethal infections and are unique in that they apparently only infect athropods. Present isolates of baculoviruses are often genus or species specific, infecting primarily lepidopteran and hymenopteran hosts. Their main advantage lies in the narrow host range and resulting compatibility with integrated pest management programmes (Payne, 1986). There are a number of commercial baculovirus products cuurently available, including "Virox", produced by Oxford Virology UK for the control of the pine sawfly and "Granupom", produced by Hoechst for the control of codling moths.

Baculoviruses are large rod-shaped, enveloped DNA viruses which can be divided into three groups on the basis of morphology; nuclear polyhedrosis virus and granulosis virus in which infective particles are packed within proteinaceous occlusion bodies late in infection and non-occluded virus which do not produce these bodies. The natural route of infection is ingestion of either occlusion bodies or virions (see Payne, 1986). The protein matrix dissolves in the gut, releasing virus particles that infect and multiply in gut epithelial cells. In Lepidoptera the infection spreads rapidly to other tissues, whereas in Hymenoptera the infection is confined to the gut. The infection usually results in the death of the host, which even with the most virulent isolates usually takes 3-4 days (see Payne, 1982, 1988). As a result the use of baculoviruses as pest control agents has been most successful when a degree of crop damage can

be tolerated as is the case with some forest pests (Payne, 1986). The main objective of the strain improvement programmes for baculoviruses is to improve their speed of action. Baculoviruses can be used as a vector for the propagation and expression of foreign genes (Smith et al., 1983) which suggests that improved baculovirus strains could be constructed. Two groups have recently cloned arthropod-specific venom genes into the genome of the baculovirus Autograph californica (Tomalski and Miller, 1991; Stewart et al., 1991). In both instances the transformed viruses had shorter LT_{50}s for their target hosts than the wild-type. A baculovirus virulence gene, known as a viral enhancing factor, has recently been cloned (Granados and Corsaro, 1990). This protein has the ability to disrupt the integrity of the insect peritrophic membrane and enhance baculovirus infection 25-fold. It is hoped that the introduction of such genes into potential pathogens or their expression in plants may result in better control of insect pests with baculoviruses.

Epizootics of entomopathogenic fungi can cause spectacular natural mortality among insect populations, demonstrating the potential of fungi for pest control (see Zimmerman, 1986). The Deuteromycotina have recieved most attention as they are relatively easy to culture and some species such as Metarhizium anisopliae and Beauveria bassiania have a broad host range. (Zimmerman, 1986; Charnley, 1989a). The Entomophthorales (Zygomycotina) also contain a large number of entomopathogenic species which are particularly prevalent in temperate climates.

Succesful field applications of Entomphthora species have been few, primarily because biopreparations often quickly loose viability (Wilding et al., 1986; Wilding, 1990).

Fungi pathogenic to insects can be found in all the major taxonomic groups. More than 700 species spanning over 100 genera have been shown to be entomopathogenic (Roberts and Humber, 1981; Wraight and Roberts, 1987). Despite the ubiquity of these fungi relatively few have been developed for pest control. Among potential targets for mycopesticides, the vine weevil Otiorhynchus sulcatus stands out in particular. This insect is a serious soil inhabiting pest of ornamental plants and soft fruit, which is difficult to control in the wake of the banning of the chemical pesticide Aldrin (Moorehouse et al., 1992a). M. anisopliae has given good control of the vine weevil in trials, when the fungus is incorporated into compost or applied as a drench (Moorehouse et al., 1990, 1992b). M. anisopliae is produced on a comparatively large scale in Brazil (under the name of "Metaquino") for the control of a number of pasture and sugar cane insects, such as the spittlebug Mahanarva postica. In China B. bassiana is produced to control pine caterpillars, Dendromliminus species, leafhoppers, Nephotettix species and the European corn borer, Ostrinia nubilalis. In Russia, B. bassiana is produced under the name of "Boverin" and is used in conjunction with reduced doses of chemical insecticides to control a number of pests, but mainly the Colorado beetle, Leptinotarsa decemlineata and the codling moth, Laspeyresia

pomonella. The use of species such as Nomuraea rileyi and Ascheronia species with more restricted host ranges would appear to be limited to where chemicals do not provide adequate control or are limited by legislation. However, such specificity can be advantageous; for example, the use of V. lecanii for whitefly control in glasshouses can be integrated with the use of Encarsia formosia without causing significant harm to the parasitoid (Gillespie and Moorehouse, 1989). V. lecanni is now available commercially as "Microgermin" (Christian Hanson Biosystems) and "Mycotal" (Koppert). For a review of the currently available fungal mycoinsecticides and of recent field trials using entomopathogenic fungi, see Zimmerman (1986) and Gillespie and Moorehouse (1989).

Several fungal products have been launched and subsequently withdrawn, often because they failed to provide reliable control (Gillespie and Moorehouse, 1989). The commercial success of a fungal pathogen is dependent on a number of factors. Gillespie and Moorehouse (1989) suggested that the following characters need to be considered during strain selection, (i) pathogenicity, (ii) spore germination and growth rate, (iii) relative humidity required for germination, (iv) effect of temperature on fungal growth, (v) sporulation on dead insects, (vi) disease spread, (vii) feasibility of production, (viii) inoculum survival during storage and (ix) fungicide resistance. Until recently it was widely believed that entomopathogenic fungi would only be used against insects whose habitat provides a suitable environment for

fungal growth, such as in soil, rice fields and in glasshouses (Gillespie and Moorehouse, 1989; Charnley, 1989a). However, this may not always be the case because it has recently been demonstrated that oil based formulations of M. flavoviride can cause infections of desert locusts and grasshoppers at 30-50°C and 30% RH (Lomer et al., 1992).

Unlike other microbial entomopathogens, fungi usually invade through the host integument, which makes them suitable candidates for the control of Hemipteran pests whose sucking mouth parts usually preclude pathogen uptake (Charnley, 1989a). A number of factors, such as the ability of fungal spores to adhere to the host, germination rate and the ability to penetrate host cuticle can affect the potential of a potential pathogen to cause disease. Particular attention has been focused on cuticle-degrading enzymes and insecticidal toxins (see Charnley, 1989b; Charnley and St. Leger, 1991), but as yet no primary determinants of pathogenicity have been established unequivocally for any entomopathogenic fungus. To date, programmes of strain improvement have concentrated on screening large numbers isolates by bioassay. However, recent advances in understanding the fungal infection process and the development of gene cloning and transformation techniques for filamentous fungi (see below) have suggested that a molecular approach to strain improvement may be possible.

Despite the potential of microbial insect pathogens for pest

control they are often overlooked in favour of chemical pesticides. The specificity of many pathogens is seen as an advantage by many scientists, but this precludes their wide marketing by commercial companies. Slow kill is also one of the constraints on the wider use of microbial pathogens. The use of molecular techniques to overcome some of these hurdles may result in the wider utilisation of these pathogens (see Payne, 1988). However, it is not clear how legislation governing the release of genetically engineered organisms will develop, but field tests using genetically engineered microorganisms including baculoviruses have been undertaken (Drahos, 1991; Bishop et al., 1989).

1.1.2. The fungal infection process.

Unlike other entomopathogenic micro-organisms, fungi rarely invade the host via the gut. Entomopathogenic fungi predominately invade their host by penetration of the external skeleton or cuticle. Most fungi are non-entomopathogenic because of their inability to degrade cuticle or overcome cuticle-based host defences (Charnley and St. Leger, 1991), but an inability to develop infection structures may also prevent a potential pathogen from establishing an infection.

The first event in the infection process is the adhesion of a fungal spore to the host cuticle. A spore must remain in contact with the host cuticle for a long enough period to germinate and

for the fungus to penetrate the cuticle (Charnley, 1984). Attachment is facilitated in V. lecanii, Hirsutella thompsonii and some Entomophthora species by the use of an amorphous mucus coat covering their conidia, whereas the dry spores of M. anisopliae, B. bassiana and N. rileyi are covered by layers of interwoven bundles of extremely hydrophobic rodlets (see Charnley, 1989b). The adhesion of the spore on the insect cuticle is multifactorial, but after the initial contact, adsorption involves electrostatic forces between the propagule and the host cuticle (Fargues, 1984) with more specific linkages occuring later. As well as the electrostatic forces mentioned, enzymes, lectins and hydrophobic bonding have all been implicated in spore adhesion (Boucias et al., 1988).

Insect cuticle consists of a relatively thin outer epicuticle (1-2 μm) and a larger procuticle (≤200 μm). The epicuticle is multilayered and consists mainly of tanned proteins, lipids, phenols and hydrocarbons. The procuticle is made up of three layers (exocuticle, mesocuticle and endocuticle) which comprises chitin fibrils embedded in a protein matrix, together with lipids and quinones (Neville, 1984). Proteins are the major components of the cuticle (some cuticles can contain ca. 70% protein). The hardness of the cuticle is dependent on the extent of tanning or sclerotisation (cross-linking by aromatic compounds such as N-acetyldopamine) of the proteins; other mechanical properties depend on the relative proportions of protein and chitin and the degree of hydration of the proteins. Thus, insect

cuticle provides a substantial barrier to potential pathogens (St. Leger, 1991).

The ability to utilise lipids that comprise the outer layer of cuticle (ie. the epicuticle) may be required for subsequent germination of the adhered spore and therefore be fundamental to pathogenesis (St .Leger, 1991). The germination *in vitro* of conidia from deuteromycete entomopathogens occurs in response to a range of non-specific carbon and/or nitrogen souces (Smith and Grula, 1981; St. Leger et. al, 1986b), whereas entomopathogenic fungi with more restricted host ranges appear to have more specific requirements for germination. For example, *Erynia variabilis* is restricted to small dipterans partly by a requirement for oleic acid to induce germination (Kerwin, 1984).

Water provides a medium for the uptake of dissolved nutrients and is therefore essential for germination. A period of soaking in water prior to the provision of a nutrient source accelerates and synchronises germination of conidia of *M. anisopliae* *in vitro* (Dillon and Charnley, 1985). Interestingly, soaked spores were significantly more virulent than controls (Hassan and Charnley, 1989). Conidial germination of *M. anisopliae*, *B. bassiana*, *V. lecanii* and some *Paecilomyces* species was optimal at relative humidities of 97-100%, with a significant decrease at 96% and no germination at all at 92% (Gillespie and Crawford, 1986).

Entomopathogens vary in the extent of surface growth prior to

penetration, but such growth is often most extensive on hard cuticle (Charnley, 1984). This may aid the location of thinner or softer cuticle, or enhance invasiveness by synergism with other hyphae (Charnley, 1984). Such growth could also be a result of the absence of required stimuli (apical swellings or appressoria on germ tubes are often a prerequisite for infection). In the case of M. anisopliae (ME1) appressoria-inducing stimuli consist of a hard surface and the presence of a low concentration of low molecular weight nitrogenous nutrients (St. Leger et al., 1989a). Appressoria are not formed in shake cultures with a variety of carbon and nitrogen sources confirming the requirement for a contact stimulus (St. Leger et al., 1989a). Evidence for such a response can also be seen in vivo; in M. anisopliae appressorial formation occurs preferentially over hair sockets of early instar larvae of Manduca sexta (St. Leger et al., 1988a), but are induced closer to the conidium on the cuticle of late instar M. sexta which lacks the extensive microfolding of the type seen on early instar cuticle (St. Leger et al., 1989b). In M. anisopliae such appressoria provide a stable platform for host penetration by attaching firmly to the host cuticle via mucilage (Zacharuk, 1970a,b). Whatever the role of appressoria in infection, appressorial formation represents a transitional step from that of germ tube to a specialised cell (the penetration peg) which breaches the integument (Zacharuk, 1970a,b).

Apart from certain exoparasitic Laboulbeniales, for example Laboulbeniopsis termitarius a parasite of subterranean termites

which derives nourishment from surface secretions (Zacharuk, 1981), most pathogenic fungi need to penetrate the host cuticle to obtain nutrients for growth and reproduction.

The epicuticle is penetrated either by infection pegs produced from the underside of appressoria or by direct entry by germ tubes (Zacharuk, 1981). Penetration occurs mainly by direct penetration of the epicuticle, particularly where there is no exocuticle, such as arthrodial membranes at joints and between segments (Charnley, 1989b; St. Leger, 1991). The mechanism for penetration of the host cuticle varies between species, but is generally considered to be a combination of mechanical pressure and enzymatic degradation (the role of cuticle-degrading enzymes is discussed below). Evidence for the physical penetration of the epicuticle is shown by the appearance of characteristic triradiate and tetraradiate fissures in the epicuticle of insects infected with some Entomophthora species (Brobyn and Wilding, 1983); however, with some interactions the presence of circular fissures at the penetration site provides evidence for enzymatic hydrolysis (Butt, 1987). After breaching the epicuticle, penetrant structures often expand laterally in the outer layers of the procuticle, producing penetration plates (Zacharuk, 1970b) or the fungus may pass through the procuticle directly (Charnley, 1984). Indentation or displacement of cuticular lamellae indicates the importance of mechanical pressure in penetrating the procuticle which is often accompanied by signs of enzymic degradation (Charnley, 1984). Partial histolysis and clearing of

the lamellar pattern around penetrant hyphae of M. anisopliae in the cuticle of M. sexta has been observed (Hassan and Charnley, 1989).

A successful infection is achieved when the fungus reaches the epidermis and finally enters the haemocoel (Charnley, 1984). Growth of the fungus within the haemocoel usually coincides with the production of blastospores and/or hyphal fragments, although some pathogens retain filamentous growth (see Charnley, 1984). Death of the insect results from either profuse growth of the fungus in the haemocoel or after relatively limited growth when toxins are presumed to cause host death (Roberts, 1980). Early host death seen with many deuteromycete pathogens is attributed to the action of such insecticidal toxins, whereas entomophthoralean pathogens usually have a longer parasitic phase with extensive growth in the haemolymph and penetration of host tissues (Charnley, 1984).

Many entomopathogens produce toxic secondary metabolites in culture; these include the cyclic depsipeptides beauvericin, bassionalide and beauverolide from B. bassiana and the destruxins of M. anisopliae (Roberts, 1980). The toxins of B. bassiana are antibiotic and behave as ionophores (Roberts, 1980). The toxins of many entomopathogenic fungi produced in culture have not been isolated from parisitised insects and their relevance to mycosis is unknown (Charnley, 1984). Destruxins have been isolated from diseased insects (Suzuki et al., 1971; Samuels et al 1988a) and

injected doses of destruxin cause incoordination, paralysis and death in Lepidopteran larvae and adult Diptera (Roberts, 1980; Samuels et al., 1988b). Samuels et al. (1988a) reported a correlation between paralysis, limited growth in the haemocoel, early death, high virulence and high destruxin production in M. anisopliae. However, two isolates which produced little destruxin were still pathogenic, but these had reduced virulence. In insects other than Lepidoptera and adult Diptera destruxins may play a part in overcoming host defences (Samuels et al., 1988a). However, the role of destruxins in pathogenesis is not clear and may not be elucidated until mutants deficient in destruxin production can be identified and complemented with cloned destruxin synthase genes (Charnley, 1990).

A number of host defence mechanisms have been identified (see Charnley, 1984, 1989b for reviews). Dillon and Charnley (1986a) reported that axenic, but not conventional locusts were susceptible to infection with M. anisopliae as a result of fungal penetration of the hindgut. This was subsequently shown to be due to the absence of toxic antifungal phenols normally produced by the gut bacterial flora (Dillon and Charnley, 1986b, 1988). Although not host derived, these toxins could offer significant protection to the host and is therefore an important factor in the symbiotic association of the host with its gut flora.

Penetration of the host cuticle often results in the deposition of oxidised phenols (melanin) around the penetrant hyphae by host

phenoloxidase. Growth and protease production by M. anisopliae was significantly reduced on melanised cuticle (St. Leger et al., 1988c). It would appear that that melanisation is primarily a defence against weak or slow growing pathogens but is relatively ineffective against more virulent pathogens (see Charnley, 1989b). The main cellular response of the insect is the encapsulation of the fungal blastospores. Activation of the prophenoloxidase cascade has been implicated in non-self recognition (see Charnley, 1989b). The final step in the system is the activation of prophenoloxidase by a trypsin-like serine protease. Protease inhibitors have been isolated from the haemolymph of a number of insects (eg. Sasaki, 1978, Eguchi et al., 1982). These inhibitors, which have a broad specificity towards fungal proteases (St. Leger, 1991), may confine prophenoloxidase activation to the site of infection and may also inhibit pathogen proteases (Charnley, 1989b).

1.1.3. Cuticle-degrading enzymes of entomopathogenic fungi.

The ability of entomopathogenic fungi to produce extracellular enzymes with activity towards the main chemical constituents of insect cuticle, namely, lipids, proteins and chitin (Neville, 1975) has been known for some years. Gabriel (1968) demonstrated that certain species of Entomophthora were capable of producing lipolytic, proteolytic and chitinolytic enzymes. Similar enzyme activities were subsequently identified in culture filtrates of B. bassiana (Leopold and Samsinakova, 1970) and M. anisopliae

(Kucera, 1980). More recently, St. Leger et al. (1986a) showed that pathogenic isolates of M. anisopliae, B. bassiana and V. lecanii produced endo- and exo- acting proteases, chitinase, N-acetyl glucosaminidase and lipase when grown in buffered cultures containing 1% ground locust cuticle as sole carbon/nitrogen source. The amounts of each enzyme produced varied considerably between strains of M. anisopliae, but high levels of endoprotease were produced by all strains, suggesting a key role for such enzymes in cuticle penetration. However, two pathogenic strains of M. anisopliae (RS 703 and RS 2134) have recently been shown to produce very little endoprotease when grown on insect cuticle in vitro (Gupta et al., 1991).

The cuticle-degrading enzymes of these deuteromycete entomopathogens are produced sequentially in culture (St. Leger et al., 1986a). Proteases and esterases appeared first (≤24 hrs), whereas chitinase and lipase did not appear until much later (3.5 and 5 days respectively). The order of appearance of the enzymes reflects the order of substrates the fungus encounters during infection, ie. epicuticular waxes followed by protein and chitin. The late appearance of lipase was apparently due to the fact that lipase is largely cell bound in young mycelia and is therefore not released until mycelial autolysis occurs in culture (St. Leger et al., 1986a). A purified alkaline protease (pI 9.5), later shown to be a chymoelastase (Pr1, see below) hydrolised ca. 30% (w/w) of cuticle proteins, releasing peptides with a mean chain length of 4.7 (St. Leger et al., 1986c) as well as a small

quantity of amino sugars. Chitinase released more N-acetyl glucosamine from cuticle than Pr1, but pre-treatment of the cuticle with Pr1 enhanced chitinase activity ca. 3.5x, suggesting that chitin is masked by protein in cuticle, which may explain the later appearance of chitinase relative to protease in culture.

Three endoproteases produced by M. anisopliae have been purified and characterised (St. Leger et al., 1987a; S. Cole, unpublished). These are a chymoelastase, Pr1, a trypsin, Pr2 and a cysteine protease with trypsin-like specificity, Pr4. Pr1 is a serine protease with a high pI (ca. 10), has high activity vs. insect cuticle and elastin, and a molecular weight of ca. 25 kDa (St. Leger et al., 1987a).In two subsequent publications Pr1 was reported to have a molecular weight of 22.5 kDa (St. Leger et al., 1989b) and 30 kDa (St. Leger et al., 1991b). St. Leger et al. (1987a) reported that a trypsin-like serine protease (Pr2), with little cuticle-degrading activity and a molecular weight of 28.5 kDa was produced as three isoenzymes, separated by isoelectric focusing at pI 4.0, 4.2 and 4.42. No evidence of these three isoenzymes was seen in a subsequent study in which Pr2 had a pI of 5.4 (S. Cole, unpublished), but a cysteine protease (Pr4) with a primary specificity for lysine and arginine residues (ie. trypsin-like) was also identified. Pr4 had a pI of 4.6, a molecular weight of 26.7 kDa and possessed relatively high cuticle degrading activity. Pr1 exhibited the greatest cuticle-degrading activity, with Pr4 possessing 51% of the cuticle-

degrading activity of Pr1. Pr2 has only 4% (St. Leger et al., 1987a) or 21% (S. Cole, unpublished) of the cuticle-degrading capability of Pr1. Pr1, Pr2 and Pr4 all have alkaline pH optima. An endoprotease with a pH optimum of 5.0-5.5 (Pr3) was identified by St Leger et al. (1987a) but not characterised further. Several isolates produce acidic proteases that occur as multiple isoenzymes and resemble Pr1 in their primary specificity but do not degrade elastin (St. Leger et al., 1987b).

Endoproteases with similar properties to both Pr1 and Pr2 have been isolated from culture filtrates of the entomopathogenic deuteromycetes M. anisopliae, B. bassiana, V. lecanii, Nomuraea rileyi and Aschersonia aleyrodris (St. Leger et al., 1987b). Despite the similarity of the substrate specificities of the chymoelastases from these organisms the enzymes of all but two strains of Metarhizium failed to cross-react (as determined by Ouchterlony immunodiffusion) with antibodies raised against Pr1 from M. anisopliae strain ME1. The widespread occurrence of such enzymes presumably reflects the importance of the enzymes in pathogenesis. A serine protease from B. bassiana produced in media with gelatin as sole carbon and nitrogen source has been purified and partially characterised (Bidochka and Khachatourians, 1987). The protease has elastolytic properties, a molecular weight of 35kDa and a pH optimum of 8.5. Endoproteases with activity vs. collagen have also been identified in culture filtrates of the entomopathogens Erynia coronata (Hurien et al., 1979) and Lagenidium giganteum (Dean and Domnas, 1983). In

contrast to the five species of deuteromycetes described by St. Leger et al. (1987b) in which Pr1 and Pr2-like activities are found as separate enzymes, the entomopathogenic entomophthoraceae E. rhizospora, E. dipterigena and E. neoaphidis produce single endoproteases with activity against trypsin and chymotrypsin substrates (Samuels et al., 1990).

Two classes of aminopeptidase have been demonstrated in filtrates of cuticle grown cultures of M. anisopliae (Charnley and St. Leger, 1989). An aminopeptidase M was found which has broad specificity with optimal activity against alanine residues, exists as multiple isoenzymes (pI 5-6). Two isoforms (pI 4.6) of a post-proline dipeptidyl aminopeptidase IV was also identified. In a separate study, an aminopeptidase which exhibits high specificity towards substrates with N-terminal L-alanyl residues was identified (S. Cole, unpublished). This enzyme, which also cleaves naphthylamides of serine and aromatic residues, exists as a dimer of two subunits of 47.8 kDa, has a pI of 4.14 and is a metalloenzyme. Alanine is the predominant amino acid in locust cuticle (St. Leger et al., 1986c). Pr1 cleaves peptide bonds after non-polar amino acids eg. alanine (elastase-like activity) and therefore would presumably release many peptides with alanine at the N-terminus, which would provide substrates for the alanine-specific aminopeptidase. Aminopeptidases with broad specificities have also been identified in filtrates of some Entomophthora species (Samuels et al., 1990).

Chitinase activity in extracellular fluids from M. anisopliae grown with chitin as sole carbon source can be separated into distinct endo- and exo-chitinase fractions (St. Leger et al., 1991a). The endo-chitinase has a pH optimum of 5.3, a molecular weight of 33 kDa and possess little or no activity against saccharides less than a tetramer, but preferentially cleaves tetrasaccharides to disaccharides (St. Leger et al., 1991a). Interestingly, the endo-chitinase was able to degrade crystalline chitin to the monomer, suggesting that the reaction proceeds via a single chain processive mechanism. The same authors also demonstrated that the N-acetyl glucosaminidase (chitobiase) had a pH optimum of 5, a molecular weight of 110 kDa, a pI of 6.4 and hydrolysed di-, tri- and tetrasaccharides to the monomer N-acetyl glucosamine. To date, no other chitinases from entomopathogenic fungi have been characterised.

The regulation of protease production has been studied in M. anisopliae and B. bassiana. In M. anisopliae both Pr1 and Pr2 are produced under conditions of carbon and nitrogen starvation (St. Leger et al., 1988b). Proteases are similarly derepressed in many Aspergillus species (Cohen, 1981). Protease production was severly impared by inhibitors of transcription and translation implying that de novo synthesis of mRNA is involved in protease regulation (St. Leger et al., 1988b). It was later shown that Pr1 transcripts appeared rapidly after transfer of mycelium to medium deficient in carbon and nitrogen (St. Leger et al., 1991b). Bidochka and Khachatourians (1988a) reported that B. bassiana

produced an extracellular protease with elastolytic properties when grown in medium with gelatin as sole carbon and nitrogen source. The protease was repressed by glucose, ammonium and N-acetyl glucosamine (Bidochka and Khachatourians, 1988a,b). St. Leger et al. (1991b) reported that V. lecanii, B. Bassiana, Tolypodocladium niveum and Paecilomyces farinosus also produced a Pr1-like enzyme during nutrient deprivation. In M. anisopliae levels of Pr1 were reported to be higher in cuticle grown cultures and Pr2 production was enhanced by BSA and cuticle (St. Leger et al., 1988a, 1991b). Gupta et al.(1991) reported that protease levels in M. anisopliae were exceptionally high when the fungus was grown with insect cuticle as sole carbon and nitrogen source suggesting that protease production may also be subject to substrate induction.

The regulation of chitinase production by M. anisopliae (St. Leger et al., 1986d) and B. bassiana (Smith and Grula, 1983) has also been studied. Chitinase synthesis in these two entompathogens is repressed by readily utilised carbon sources, but under derepression conditions is induced by N-acetyl glucosamine, the major monomeric constituent of chitin. N-acetyl glucosaminidase production by M. anisopliae was less susceptible to catabolite repression than chitinase and basal levels were only slightly enhanced by N-acetyl glucosamine (St. Leger et al., 1986d). Chitinase production in the mycopathogenic fungi Trichoderma harzanium (Ulhoa and Peberdy, 1991) and Aphanocladium album (Blaiseau et al., 1992) has also been shown to be regulated

by similar inducer-repressor mechanisms.

Despite the ubiquity of cuticle-degrading enzymes among entomopathogenic fungi and their extensive study *in vitro* the role of such enzymes in pathogenesis is still unclear (see Charnley, 1989b; Charnley and St. Leger, 1991). Endoprotease and aminopeptidase activity was detected in extracts of blowfly wings infected with *M. anisopliae* (St. Leger et al., 1987c). The endoprotease activity separated into two fractions with similar activities to Pr1 and Pr2, with the presence of Pr1 proven by immunodiffusion. Protease was also detected histochemically on conidia, on host cuticle and during all stages of germination. High levels of protease (demonstrated histochemically or extracted from infected wings) was only associated with the formation of large numbers of appressoria (St. Leger et al., 1987c). It was subsequently shown by incorporation of ^{35}S-methionine into differentiating hyphae and western blot analysis that Pr1 is the major protein synthesised by differentiated germlings of *M. anisopliae* and that Pr1 is the major protein produced during infection of host cuticle (St. Leger et al., 1989b). Also, Pr1 transcripts were not detected in conidia and only appeared when the formation of infection strucures was induced by nutrient deprivation (St. Leger et al., 1991b). Goettel et al. (1989) using a protein-A immunogold labelling technique were able to demonstrate that Pr1 was secreted by appressoria on the host cuticle and by penetrant hyphae within the cuticle. During the early stages of infection Pr1 was

confined to the region immediately surrounding the hyphae, but diffused throughout the cuticle later in infection. The production by Metarhizium of high levels of Pr1 during differentiation and host infection suggests a key role for Pr1 in cuticle penetration. St. Leger et al. (1988a) reported that inhibition of Pr1 (with turkey egg white inhibitor or anti-Pr1 IgG) during infection of M. sexta prevented early penetration of host cuticle and significantly delayed development of symptoms and mortality. Inhibition of Pr1 also reduced cuticle melanisation (a host response to infection) and invasion of the haemolymph. The fact that turkey egg white inhibitor did not affect germination rate, growth of the fungus on the cuticle surface or appressorial formation led the authors to propose Pr1 as a pathogenicity determinant of M. anisopliae.

The role of chitinase in cuticle penetration is less clear. N-acetyl glucosamine, but not endochitinase was extracted from blow fly wings infected with M. anisopliae (St. Leger et al., 1987c). Also, the products of chitin hydrolysis were not detected in comminuted infected cuticle; this suggests that endochitinase may not be involved in initial cuticle penetration, but may play a nutritional role in the saprophytic stage of the infection. N-acetyl glucosamine may hydrolyse the chitin fibrils exposed by the proteases and in turn release the monomeric inducer of endochitinase. However, Hassan and Charnley (1989) reported that the cuticle of Dimilin-treated insects (Dimilin inhibits chitin synthesis in insects) afforded little resistance to penetrant

hyphae of M. anisopliae and was completely destroyed by enzymatic hydrolysis during the process. This suggests that as chitin represents such a significant barrier to infection endochitinase may play a role in cuticle penetration.

The disappearance of the wax layer beneath appressoria of M. anisopliae on Elaterid (click beetle) cuticle suggests the action of esterase and/or lipase (Zacharuk, 1970b). Coincidentally, esterase but not lipase was extracted from blow fly wings infected with M. anisopliae (St. Leger et al., 1987c). However, potential substrates for lipases and esterases form only a minor part of the extractable cuticular lipids casting doubt over the role of these enzymes in penetration (see Charnley and St. Leger, 1991).

1.1.4. Determinants of pathogenicity and/or virulence.

There are evidently many factors involved in the fungal pathogenesis of insects; failure or poor performance of a particular strain at any stage of the infection process could result in a loss of pathogenicity or reduced virulence. Failure of the fungal spore to adhere to and germinate on the host cuticle would obviously overide any other potentially virulent traits of a potential pathogen. Al-Aidroos and Roberts (1978) reported that very few conidia of a non-pathogenic mutant of M. anisopliae adhered to the larval syphon of Culex pipiens compared to the wild-type. The same authors also reported that highly

virulent strains had increased in-situ germination rates. Similar results have been described for V. lecanii (see Heale et al., 1989) where more virulent isolates displayed the most rapid in vivo germination. A correlation between germination rate and virulence was not apparent in vitro presumably because of the importance in vivo of a tolerance to toxic substances on the cuticle surface (see Charnley, 1989b; St Leger, 1991).

As described previously, there is very strong evidence for cuticle-degrading enzymes as determinants of virulence from studies by St. Leger and co-workers on M. anisopliae. However, some other reports have been conflictory: Jackson et al. (1985) reported high extracellular protease activity in 18 wild-type strains of V. lecanii irrespective of pathogenicity towards the aphid Macrosiphoniella sanborni. A similar lack of correlation between protease activity and virulence was reported for M. anisopliae during infection of the planthopper Niloparvata lugens (Samuels et al., 1989). However, all highly virulent isolates produced chitinase, lipase and protease. Both endo- and exo-chitinases are produced by two virulent and one avirulent isolates of N. rileyi (El-Sayed et al., 1989). However, high levels of both enzymes were only detected in the virulent isolates, with the greatest difference occuring at germination. A similar correlation between chitinase and virulence has been reprted in V. lecanii (Jackson et al., 1985) and B. brogniartii (Paris and Ferron, 1979). This contrasts with the findings of St. Leger et al (1987b) who failed to find evidence for the

involvement of endochitinase during infection of blowfly wings with M. anisopliae. However, these studies which looked for correlations between particular traits and virulence may be less reliable than the experimental approach of St. Leger and co-workers, as the wild-type isolates used are of very different genetic backgrounds. It is apparent that the role of cuticle-degrading enzymes is variable for each host-parasite interaction. St. Leger (1990) has suggested that proof for the involvement of enzymes in cuticle penetration would require that (i) the enzyme must be present at the site of penetration; (ii) enzyme deficient mutants (as yet to be identified) must be avirulent; (iii) subsequent expression of the enzyme gene (by transformation) should restore pathogenicity and (iv) specific inhibitors should block the penetration step and protect insects from disease.

The production of blastospores, protoplasts or hyphal bodies within the haemocoel may contribute to the avoidance by the pathogen of cellular host responses (see Charnley, 1989b). A failure to do so may result in a more vigorous host response and subsequent failure of the pathogen to kill the host. Toxin production at this late stage in the infection process often results in early host death. The ability of an isolate to produce large amounts of such insecticidal toxins may give rise to a more virulent pathogen. Such a correlation between destruxin production and virulence has been reported for M. anisopliae during infection of M. sexta (Samuels et al., 1988a).

1.1.5. Strain improvement.

To date, the main effort towards obtaining more virulent strains of entomopathogenic fungi has been to screen large numbers of isolates by bioassay. As our understanding of the pathogen-host interaction improves and determinants of pathogenicity and/or virulence identified, then more rational approaches to strain improvement can be undertaken. It is likely that different pathogenic isolates will contain different virulence determinants, therefore it may be possible, using some form of genetic manipulation, to combine such desirable traits in a single isolate to produce a hypervirulent pathogen.

Deuteromycetes (such as Metarhizium) lack a conventional sexual cycle and therefore genetic recombination can only be achieved via the parasexual cycle or by genetic engineering (Heale et al., 1989; Clarkson, 1992). The essential steps in achieving recombination via the parasexual cycle are the formation of a heterokaryon by hyphal anastomosis or protoplast fusion followed by rare nuclear fusions to produce novel genotypes, which can arise through mitotic crossing over in diploid nuclei and through the random loss of chromosomes during haploidisation. Heale et al. (1989) reported the combination of high virulence and enhanced sporulation in a cross between two strains of M. anisopliae. Unfortunately, parasexual recombinants often express a reduction in virulence in comparison with the wild-type parents (Heale et al., 1989). In addition, genetic exchange between

unrelated strains via hyphal anastomosis is frequently restricted by vegetative incompatibility and this may constitute a major barrier to strain improvement in some entompathogenic fungi (Clarkson, 1992). However, vegetative incompatibility may sometimes be overcome through the use of protoplast fusion (eg. Jackson and Heale, 1987).

The relatively recent development of gene cloning and transformation techniques for filamentous fungi (see below) could enable the investigation of pathogenicity determinants at the molecular level. Potential pathogenicity/virulence determinants that are single gene products, such as the chymoelastase, Pr1 and destruxins (similar toxins are synthesised by large multi-functional enzymes) of M. anisopliae lend themselves to this approach. Molecular techniques could be used to increase the copy number and/or alter the expression of such genes as well as providing a tool for moving genes between species (Clarkson, 1992) with the hope of engineering more virulent strains for use in the biological control of insect pests.

1.1.6. Molecular cloning and transformation in filamentous fungi.

The genetic engineering of filamentous fungi is dependent on the ability to introduce and express exogenous DNA. Such transformation procedures for filamentous fungi have been reported many times using a variety of selectable markers (see Hynes, 1986; Fincham, 1989). DNA-mediated transformations are

often based on the addition of DNA to cells in the presence of Ca^{2+} followed by the addition of polyethylene glycol (see Fincham, 1989), although the transformation of whole cells (eg. conidia) has been reported using lithium acetate (Dhawale et al., 1984). Transformation has been achieved by complementation of auxotrophic mutants (eg. Ballance et al., 1983; John and Peberdy, 1984) with homologous genes and by the use of dominant selection markers, such as resistance to benomyl (eg. Orbach et al., 1985) and Oligomycin (Ward et al., 1986) or using the amdS gene which confers the ability to utilise acetamide as sole nitrogen source (eg. Kelly and Hynes, 1985). Transformants usually arise at a low frequency (0.1-10 transformants per μg of vector DNA) through the random integration of the DNA into the genome, although homologous integration can occur (Fincham, 1989). There have been some reports of autonomously replicating DNA sequences (eg. Van Heeswijk and Roncerno, 1984; Powell and Kistler, 1990; Gems et al., 1991) which have resulted in a marked increase in transformation frequency (100-1000 transformants per μg vector DNA).

The unavailability of auxotrophic markers and the fact that M. anisopliae is resistant to high concentrations of the antibiotics hygromycin B, geneticin, oligomycin and phleomycin (Bernier et al., 1989) precludes the use of many cloned genes which have been used to transform other filamentous fungi. However, M. anisopliae has been transformed to benomyl resistance with the β-tubulin genes from N. crassa (Bernier et al., 1989) and A. nidulans

(Goettel et al., 1990), but the transformation frequencies in each case were very low (0.1 transformants per μg DNA). A nitrate reductase mutant of the entomopathogen B. bassiana has also been transformed with the nitrate reductase (niaD) gene of A. nidulans (Daboussi et al., 1989).

Many genes from filamentous fungi have now been cloned, often utilising similar stratagies to those developed for other organisms (see Rambosek and Leach, 1987; Gurr et al., 1987). Early attempts at cloning genes from filamentous fungi involved the complementation of auxotrophic mutants of E. coli, for example the trpC gene of A. nidulans (Yelton et al., 1984). However this technique is problematic due to the fact that fungal promoter and termination signals may not be recognised by E. coli (Rambosek and Leach, 1987). This can be solved by complementation in the homologous system, when theoretically any gene can be cloned for which a corresponding mutant exists provided a good transformation system is available. Such examples are the leu gene of Mucor. circinelloides (van Heeswijk and Roncerno, 1984) and the isocitrate lyase (acuD) gene of A. nidulans (Ballance and Turner, 1986). Since transformation in filamentous fungi usually results in the integration of the vector into the genome any complementary cloning method necessitates subsequent marker rescue, which usually involves digestion the transformed DNA, followed by circularisation of the plasmid and transformation of a suitable E. coli host. However, examples have been reported of the recovery of plasmids in E. coli without the prior digestion

of the DNA (see Turner and Ballance, 1985; Rambosek and Leach, 1987). Other techniques which have been used to clone filamentous fungal genes include differential hybridisation (eg. Hynes et al., 1983), antibody screening of expression libraries (eg. Hohn and Beremand, 1989; Blaiseau et al., 1992) heterologous hybridisation between genes of two species (eg., Orbach et al., 1985), the generation of gene specific probes using the polymerase chain reaction (eg. Scott-Craig et al., 1990) and the use of synthetic oligonucleotide probes deduced from amino acid sequences (eg. Kinnaird et al., 1982). To date no genes from entomopathogenic fungi have been cloned.

2. MATERIALS AND METHODS

2.1. MATERIALS

All chemicals were purchased, unless otherwise stated, from either Sigma or BDH. Culture media were purchased from Lab. M. Restriction enzymes, polynucleotide kinase and other DNA modifying enzymes were obtained from Northumbria Biologicals Limited and used in the recommended reaction buffers. Radiolabelled nucleoside triphosphates, amino acids, protein molecular weight markers and blotting membranes were purchased from Amersham Int. Ampholines, ion-exchange and gel filtration matrices were from Pharmacia. HPLC grade solvents were purchased fron Rathburns.

2.1.1. Locust culture.

Stock cultures of Schistocerca gregaria maintained in metal cages in a controlled environment insectary at 30°C ± 3°C, 25-30% RH and under a photoperiodicity of 11 hrs light, 13 hrs dark. The locusts were fed daily with wheat shoots and bran supplemented with dried brewers' yeast.

2.1.2. Preparation of locust cuticle.

Bulk preparations of cuticle were prepared from adult desert locusts (Schistocerca gregaria). Approximately 100 locusts were

homogenised in a Waring blender in a solution of 1% potassium tetraborate. The cuticle pieces were then washed extensively in dH_2O, stirred overnight in 1% potassium tetraborate and then dried at 37°C. The cuticle pieces were then milled to a fine powder in a Glen-Creston hammer mill (DEH 48) using the 0.5 mm sieve. The powder was then washed in 1% potassium tetraborate and finally in dH_2O, allowed to settle and impurities removed by dicarding any floating material. This method gives a pure cuticle preparation and does not extract cuticle proteins or phenols (Andersen, 1980).

Protein was removed from cuticle by hydrolysing the cuticle powder in 30% (w/v) KOH at 80°C for 2 hrs. The chitinous cuticle, which is resistant to KOH treatment was then removed from the hydrolysate, washed extensively in dH_2O and dried. (Grosscurt, 1978))

Hydrocarbons, glycerides and sterol esters were removed from cuticle by stirring the powder in ether or chloroform for 2 hrs at room temperature. The remaining cuticle was then stirred as above in ethanol to extract phospholipids and fatty acids (Gilby, 1980), washed in dH_2O and dried.

2.2. CULTURE CONDITIONS AND GROWTH ASSESSMENT

2.2.1. Fungal isolate; maintenance and culture.

M. anisopliae isolate ME1 (origin: pecan weevil),originally obtained from Tate and Lyle, Group Research and Development Laboratory, Reading, U.K. was used exclusively throughout this study. Previous work in this laboratory has shown ME1 to be a representative isolate in terms of pathogenicity and production of cuticle degrading enzymes and destruxins (St. Leger et al.,1986c, 1987b, 1987b; Samuels et al.,1988a). The virulence of this isolate towards the desert locust S. gregaria and the tobacco hornworm Manduca sexta was regularly tested in this laboratory.

The fungus was maintained routinely on 1/4 strength Sabouraud's Dextrose Agar (SDA) containing 0.25 % yeast extract at 27°C. Stock cultures were maintained on 1/4 SDA at 4°C and subcultured every 6 months.

Conidial suspensions were prepared from actively growing cultures on 1/4 SDA by washing with 0.05% Tween 80. The resulting suspension was centrifuged at 3000g for 5 mins. and then resuspended in sterile distilled water (dH_2O).

Liquid cultures were grown in 250 ml Erlenmeyer flasks containing 1% (w/v) ground cuticle in 100 ml the modified basal salts medium

described by Cooper and Wood (1975), which contained per litre, 1g KH_2PO_4, 0.5g $MgSO_4.7H_2O$ and 10 ml trace element solution (KH_2PO_4 [0.1% w/v], $MgSO_4.7H_2O$ [0.05], $FeSO_4.7H_2O$ [0.2], $ZnSO_4.7H_2O$ [1], $NaMoO_4.2H_2O$ [0.02], $CuSO_4.5H_2O$ [0.02], $MnCl_2.4H_2O$ [0.02]) plus 0.05M 4-morpholineethane sulphonic acid (MES) pH 6, or in complete medium (modified Czapek Dox supplemented with casein hydrolysate, malt extract, yeast extract and mycological peptone at 0.2% [w/v]). The flasks were inoculated with 4 x 10^6 spores (unless otherwise stated) and incubated on an orbital shaker at 150 rpm at 27°C.

Carbon sources were usually supplied at 1% (w/v). For certain experiments amino acid monomers and dimers were also supplied to liquid cultures in a restricted manner by means of diffusion capsules (Pirt, 1971; Cooper and Wood, 1975). Rates were controlled by altering the concentration of amino acids inside capsules and the number of membranes (dialysis tubing) through which diffusion occurred. Capsules containing alanine (10%, w/v) and three membranes or alanine dimer (5%, w/v) and one membrane gave diffusion rates of approximately 20 $\mu g\ ml^{-1}hr^{-1}$ (see appendix 2).

Culture media were sterilised by autoclaving at 121°C, 0.1 MPa pressure for 15 mins. Small volumes of liquid (eg. amino acid solutions) were sterilised by membrane filtration (0.2 μm, Oxoid membrane filters). Empty diffusion capsules were washed in absolute ethanol, and then autoclaved with carbon and nitrogen

sources at 115°C, 0.1MPa pressure for 10 mins. before placing in cultures. Insoluble carbon and nitrogen sources were sterilised in an atmosphere of propylene oxide.

2.2.2. Transfer experiments: Enzyme production by derepressed mycelium.

100 ml of complete medium in 250 ml Erlenmeyer flasks was inoculated with 4 x 10^6 conidia and incubated in an orbital shaker at 150 rpm at 27°C for 3-days. The mycelium was then retained on two layers of sterile muslin, washed with basal salts medium and transferred to 100 ml of basal salts medium containing 0.05M MES and incubated for a further 24 hrs. Potential inducers of protease production were then added and protease activity assayed for up to 24 hrs.

2.2.3. Fungal growth assessment.

1. Measurement of mycelial dry weight.

Mycelium from liquid cultures was collected over a Buchner funnel and the mycelial mat dried to a constant weight at 70°C in a pre-weighed piece of aluminium foil. The dried mycelium was cooled to room temperature in a desiccator before weighing.

2. Ergosterol extraction from mycelium.

The procedure used for the extraction and quantification of ergosterol was a modification of the method described by Seitz et al. (1979). Mycelium from 1 flask of liquid culture was collected over a Buchner funnel and homogenised for 1 minute on full power on a "Pulse Matic 16" osterizer in 40 ml of methanol : dichloromethane (2:1 v:v). After 30 mins. the suspension was filtered over a Buchner funnel and the residue washed with 40 ml of dichloromethane and then 40 ml of 2 M KCl in 0.5 M potassium phosphate buffer (pH 7.4). The filtrate and the washings were combined and the aqueous phase discarded. The organic phase was evaporated to dryness under reduced pressure at 40°C in a rotary evaporator. The solid was saponified in 30 ml 1 M KOH in 95% ethanol (v:v) for 1 hr at 70°C. Once cool, 60 ml of distilled H_2O was added and the non-saponified fraction extracted with three 100 ml washings of petroleum ether. The three washings were combined and evaporated to dryness under reduced pressure at room temperature in a rotary evaporator. The solid obtained was stored under nitrogen in sealed tubes at 4°C until required.

2.2.4. Fast atom bombardment mass spectrometry.

A VG Analytical 70 70E mass spectrometer was utilised with a mass spectrometry services data system to obtain mass spectra of standard and extracted ergosterol. Xenon gas was used in the atom gun running at 8 kV with 3-nitro benzyl alcohol as the matrix.

2.3. ENZYMOLOGY

2.3.1. Enzyme assays.

The endoproteases Pr1 and Pr2 were assayed by monitoring the release of nitroanilide at 410 nm from the substrates Suc-$(Ala)_2$-Pro-Phe-NA and Bz-Phe-Val-Arg-NA respectively in 0.1 M Tris-HCl, pH 8 over 3 min. at room temperature (St. Leger et al. 1987a). Pr4 was assayed as for Pr2 except PMSF was added to the filtrate at 10 mg/ml and incubated for 30 mins. at room temperature prior to the assay. The 1 ml reaction volume, contained 925 μl buffer, 25 μl enzyme solution and 50 μl substrate (2 mM in DMSO). Enzyme activity is expressed as nmol nitroanilide released ml^{-1} min^{-1}. An extinction coefficient (Σ) of 8800 at 410 nm was used (Erlanger et al., 1961).

Alanyl-aminopeptidase was assayed by hydrolysis of alanine β-naphthylamide and measured by coupling the released β-naphthylamide to p-dimethylaminocinnamaldehyde (Nakadai et al., 1973). The hydrolysis was performed by the addition of 100 μl filtrate to 1 ml of 2mM amino acyl β-naphthylamide in Britton-Robinson universal buffer pH 7 at 40°C. The reaction was terminated and the product determined with 1 ml 0.7% (v:v) HCl in ethanol followed by 1 ml 0.06% (w:v) p-dimethylamino-cinnamaldehyde in ethanol. The reaction was allowed to stand for 10 mins. before measuring the absorbance at 540 nm.

2.3.2. Enzymatic characterisation using the Api Zym system.

The enzymatic profiles of cultures were studied using the semi-quantitative API-ZYM system (Slots, 1981). The 19 enzymes and their substrates are listed in Table 1.
60 µl of dialysed culture filtrate was added to each of the 20 cupules of the API-ZYM gallery which was then placed in a moist chamber at 30°C for 3 hrs. After incubation, 30 µl of reagent ZYM A (25% Tris, 4.07% HCl, 10% SDS) and reagent ZYM B (0.35% Fast blue BB in 2-methoxyethanol) were added to each cupule. The colour reaction was read after 10 mins.

The results were recorded by assigning arbitrary values 0-5 to the colours developed as per the colour chart provided by the manufacturers (API). Zero corresponds to a negative reaction, 5 to a reaction of maximum intensity.

2.3.3. Enzyme purification techniques.

Culture filtrates were reduced in volume by ultrafiltration on YM5 (Amicon) membranes (5 KDa exclusion) prior to column isoelectric focusing (IEF) or High-performance liquid chromatography (HPLC).

Preparative IEF was performed on a 440 ml apparatus (LKB) utilising a sucrose density gradient containing 2% (w/v) ampholines (pH 3.5 - 10, Pharmacia) The column was run at 1 kV for 16 hours at 6°C. The sucrose and ampholines were subsequently

Table 1.

Enzymes detectable by the API ZYM test

No.	Enzyme assayed	Substrate
1	Control	
2	Phosphatase alkaline	2-naphthyl phosphate
3	Esterase (C 4)	2-naphthyl butyrate
4	Esterase lipase (C 8)	2-naphthyl caprylate
5	Lipase (C 14)	2-naphthyl myristate
6	Leucine aminopeptidase	L-leucyl-2-naphthylamide
7	Valine aminopeptidase	L-valyl-2-napthylamide
8	Cysteine aminopeptidase	L-cystyl-2-napthylamide
9	Trypsin	N-Bz-DL-arg-2-napthylamide
10	Chymotrypsin	N-glutaryl-phe-2-napthylamide
11	Phosphatase acid	2-naphthyl phosphate
12	Phosphoamidase	Naphthol-AS-BI-phosphate
13	α galactosidase	6-Br-2-naphthyl-αD-galactopyranoside
14	β galactosidase	2-naphthyl-βD-galactopyranoside
15	β glucuronidase	Naphthol-AS-BI-βD-glucuronide
16	α glucosidase	2-haphthyl-αD-glucopyranoside
17	β glucosidase	6-Br-2-naphthyl-βD-glucopyranoside
18	N-acetyl-β glucosaminidase	1-naphyl-N-acetyl-βD-glucosamine
19	α mannosidase	6-Br-2-naphthyl-αD-mannopyranoside
20	α fucosidase	2-naphthyl-αL-fucopyranoside

removed from fractions of interest by gel filtration.

Gel filtration was performed on a column (90 x 2.4 cms) of Sephadex G100 equilibrated and developed in 0.1 M NaCl.

Ion-exchange chromatography was performed on a column (10 x 1 cms) of Q-Sepharose equilibrated in 10 mM Tris.HCl pH 8. Before loading the sample, the column was first washed with 30 ml equilibration buffer and the proteins then eluted with a linear 60 ml gradient between 0.01 and 0.75 M Tris pH8.

All HPLC was performed on a Gilson system comprising two 303 pumps, a 811B dynamic mixer, a 401 dilutor, a 231 sample injector, a Rheodyne injection valve fitted with either a 20 or 100 μl loop and a Gilson 118 UV dual wavelength detector. The system was controlled by the Gilson 714 manager on an Opus computer. The columns and mobile phase conditions used are described with the experiments.

Protein concentrations were determined by the Coomassie Blue binding assay (Bradford, 1976) as supplied by Bio-Rad.

2.3.4. Generation of tryptic fragments of Pr1.

10 mg of purified Pr1 in 0.5 ml dH_2O was first boiled for 10 mins., cooled and the denatured, inactivated enzyme then

incubated for 6 hrs at 37°C with 50 μg of trypsin (bovine pancreas, Sigma).

2.3.5. Polyacrylamide gel electrophoresis (PAGE) of proteins.

Proteins were separated on the basis of molecular weight by SDS-PAGE using the discontinuous buffer system as described by Laemmli and Farre (1973).

10% acrylamide gels 1.5 mm thick were used where protein detection was by Coomassie Blue. Gels dried for autoradiography were 0.75 mm thick. Gels were run at a constant voltage of 150 V until the dye front reached the separating gel when the voltage was increased to 250 V. The molecular weight markers for Coomassie stained gels were bovine albumin, egg albumin, pepsin, trypsinogen, β-lactoglobulin and lysozyme (molecular weights 66, 45, 34.7, 24 and 14.3 kDa respectively as supplied by Sigma (marker kit SDS-6H). For gels dried for autoradiography ^{14}C-methylated proteins lysozyme, trypsin inhibitor, carbonic anhydrase and ovalbumin (molecular weights 14.3, 21.5, 30 and 46 respectively) as supplied by Amersham Int. (kit no. CFA 755).

2.3.6. TCA precipitation of proteins.

Prior to PAGE protein samples were desalted by TCA precipitation. TCA was added to give 10% (w/v) and the samples incubated on ice for 30 mins. The proteins were recovered by centrifuging at

13,000 g in a microfuge, washed 3 times in ethanol:di-ethyl ether (50:50), dried and then resuspended in loading buffer.

2.3.7. Protein detection in polyacrylamide gels.

Staining with Coomassie Blue:

Gels were gently shaken in approximately 500 ml of 25% methanol, 10% acetic acid containing 0.1% Coomassie Blue R250 (LKB) overnight at room temperature. Gels were then destained in 25% methanol, 10% acetic acid.

Staining with silver:

Gels were silver stained using a modification of the method described by Sammons et al. (1981). Gels were stained with silver after staining with Coomassie Blue as described above. After destaining, the gels were washed in 10% ethanol containing 0.5% acetic acid for 1 hr. The gel was then equilibrated in a solution of 0.19% silver nitrate for 2 hrs before washing in " Milli- Q" water for at least 2 minutes to reduce background. The gel was then placed in a reducing bath of 0.75 M NaOH, 87.5 mg/ml $NaBH_4$ and 7.5 ml/l 37% formaldehyde until bands were clearly visible: The gels was then placed in dH_2O.

2.3.8. Protein sequencing.

All sequencing of proteins and peptides was performed on an Applied Biosystems 477A protein sequencer by the Protein Sequencing Facility, Dept. of Biochemistry, University of Cambridge.

2.3.9. Ninhydrin assay for amino groups.

The amino acid concentration of culture filtrates was estimated using the ninhydrin assay of Moore and Stein (1948). 100 μl of supernatant was added to 1 ml of ninhydrin reagent (prepared immediately before use by mixing equal volumes of 4% [w/v] ninhydrin in 2- methoxyethanol and 0.2% $SnCl_2$ in 0.2M citrate pH 5) and subsequently boiled for 20 mins. The tubes were then cooled and 3 ml of 50% (v/v) isopropanol added prior to measuring the absorbance at 570 nm. The results are expressed as μg alanine equivalents ml^{-1} (see appendix 1).

2.4. MOLECULAR BIOLOGY

2.4.1. DNA Manipulations.

All standard DNA manipulations were performed as described in Maniatis et al. (1982) and Sambrook et al. (1989).

Agarose gels were made using "ultra pure" electrophoresis grade

agarose (BRL), in Tris-acetate buffer (Maniatis et al.,1982). Sample loading buffer consisted of 25% ficoll (type 400), 5mM EDTA and bromophenol blue at 0.25% as tracking dye. DNA was visualised by incorporating ethidium bromide into the gel and running buffer at 0.5 μg/ml.

2.4.2. Fungal RNA extraction.

The RNA extraction method was a modification of a method described by Chambers and Russo (1986): Mycelium was prepared by either inoculating 100 ml of modified basal salts medium (Cooper and Wood, 1975) containing 1% ground cuticle in 250 ml erlenmeyer flasks with 10^7 conidia and incubating at 150 rpm for 3-7 days at 27°C, or by transfering mycelium from 3 day old CM cultures to basal salts medium as described in section 2.2.2.

Mycelium was collected over a Buchner funnel and then 10g of mycelium was ground to a fine powder under liquid nitrogen and suspended in 100 ml of 6 M urea, 3 M LiCl. The suspension was homogenised for 1 min. in a "pulse matic 16" osterizer and then centrifuged at 13,000g for 20 mins. at 4°C. The supernatant was kept at -20°C overnight to precipitate the RNA. The RNA was recovered by centrifugation as above and the pellet resuspended in 10 ml of 0.5% SDS, 10 mM Tris-HCl pH 7.5, 1 mM EDTA. The solution was extracted once with phenol/chloroform and once with chloroform. The RNA was precipitated with 0.1 vols. Na acetate pH 5.2 and 2 vols. ethanol overnight at -20°C. The RNA was recovered

by centrifugation as above, washed gently with 70% ethanol, dried and resuspended in dH_2O.

2.4.3. Purification of messenger RNA (mRNA).

1. Messenger RNA for use in <u>in vitro</u> translations and cDNA cloning was purified using Hybond messenger affinity paper (mAP, Amersham Int.). 1 cm^2 of mAP was wetted with 20 μl of 0.5 M NaCl then air dried on filter paper. RNA in 20-40 μl of H_2O was heated to 65°C, chilled on ice and 0.1 vols. 5 M NaCl added. The RNA was spotted onto the mAP and left to adsorb for 15-20 mins. The mAP was then washed twice in 0.5 M NaCl for 5 mins, and twice in 70 % ethanol for 5 mins. and then air dried. The RNA was recovered by heating the mAP to 65°C in 200 μl dH_2O for 5 mins. and then vortexing briefly. The mAP was then removed and the RNA precipitated with 2 vols. ethanol overnight at -20°C. The mRNA was recovered by centrifugation in a microfuge for 10 mins. The pellet was washed with 70% ethanol, dried and resuspended in 20 μl of dH_2O.

2. mRNA used to synthesise cDNA for polymerase chain reaction experiments was purified using a poly (A) Quik purification kit (Stratagene) as described by the manufacturer.

2.4.4. <u>In vitro</u> translation of mRNA.

<u>In vitro</u> translation of mRNA was performed using a complete

rabbit reticulosyte lysate, supplemented with amino acids minus methionine (Amersham Int.). 8 μl of lysate was incubated with 100 ng of mRNA and 1 μl ^{35}S methionine (800 Ci $mmol^{-1}$, Amersham Int.) for one hour at 30°C. The products were separated by SDS-PAGE, and visualised by autoradiography at -70°C overnight using β-max hyperfilm (Amersham Int) after drying the gel.

2.4.5. Raising and purification of antibodies vs Pr1.

Antibody vs Pr1 was raised using a modification of the method devised by Stevenson (1974) and essentially as described by St. Leger et al. (1987c). Due to the toxic nature of Pr1 the protease was first adsorbed onto an ovoinhibitor-Sepharose complex.

2 g of CnBr-activated Sepharose was swollen in 50 ml of 1 mM HCl for 15 mins., washed over a sintered glass funnel with 200 ml of 1 mM HCl and suspended in 6 ml coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl pH 8.3). Ovoinhibitor (5 mg/ml Sepharose) was dissolved in coupling buffer and added to the Sepharose and agitated at room temperature for 2 hrs. The gel was then washed with 10 vols. of coupling buffer. The gel was suspended in 20 ml 0.1 M Tris-HCl pH 8 and incubated at room temperature for 2 hrs to block any unreacted groups. The Sepharose was then washed with 5 vols. of coupling buffer, then 0.1 M sodium acetate, 0.5 M NaCl pH 4 and then again with coupling buffer. The gel was then suspended in 2 ml 50 mM phosphate, 0.5 M NaCl pH 7, adsorbed with 3 mg Pr1 and stored at -20°C in three 1 ml samples.

One sample was mixed with an equal volume of Freund's complete adjuvant and injected intramuscularly into the thigh of a rabbit. This was repeated after 2 and 4 weeks. After 6 weeks the rabbit was injected intra-venously with 1 mg of Pr1 denatured with SDS. Blood was taken 10 days after the final injection and incubated at 37°C for 1 hr and then 4°C for 1 hr before serum separation by centrifugation at 2,500g.

IgG antibodies were purified by ammonium sulphate precipitation and ion exchange chromatography as follows: Ammonium sulphate to 50% saturation (290 g/l) was added at 0°C to serum and the proteins recovered by centrifugation at 10,000g for 10 mins. Albumin, transferrin and other contaminating proteins were removed by successive washing of the pellet until it was white. The pellet was dissolved in 10 mM Na_2HPO_4, pH 8 and dialysed overnight against dH_2O. Precipitated lipoproteins were removed by centrifugation at 10,000g for 10 mins. and the supernatant dialysed overnight against 10 mM Na_2HPO_4, pH 8. The serum was then loaded onto a DEAE-Cellulose column (4 ml gel/ml serum) previously equilibrated with the same buffer. The column was washed with 2 column volumes of the same buffer and the eluted IgG concentrated by ammonium sulphate precipitation as above. The pellet was dissolved in and dialysed against 10 mM Tris-HCl, 150 mM NaCl pH 7.4.

2.4.6. Ouchterlony double immunodiffusion.

The titre and specificity of the antiserum was tested by Ouchterlony double immunodiffusion. The diffusion was carried out in 1% agarose gels containing 50 mM phosphate, 100 mM NaCl, pH 7.2 cast on microscope slides. Wells were formed by removing agarose plugs with a gel punch connected to a vacuum. Wells were filled with 10 μl of antigen or antiserum and left to diffuse overnight at room temperature. The precipitin lines were visualised by pressing the gels between filter paper, washing in 0.1 M NaCl (3 x 15 mins.) and staining with Coomassie Blue as described in section 2.3.7..

2.4.7. Immunoprecipitation of <u>in vitro</u> translation products.

The method was a modification of that described by Kessler (1981). 3 vols. of a solution containing 1 M NaCl and 1% Triton-X 100 was added to the translation products. After mixing, 60 μl of Protein-A sepharose supension (125 mg ml^{-1} in 25 mM Tris.HCl pH 7.5, BSA 1 mg ml^{-1}) plus 10 μl pre-immune serum (samples taken from rabbits prior to inoculation) were added and the mixture incubated on a rotating wheel at 4°C for 2 hrs. The samples were centrifuged for 4 mins. at 13,00g in a microfuge, the supernatant removed and the pellet washed 4x with 67 μl of 0.75 M NaCl, 1% Triton-X 100. The supernatant and the washings were pooled to which 60 μl of the Protein-A sepharose suspension and 10 μl of immune serum were added and the samples again incubated on a

rotating wheel at 4°C overnight. The samples were centrifuged as before, the supernatant discarded and the pellet washed 4x with 200 µl of 0.75 M NaCl, 1% Triton-X 100. The precipitated protein antibody complex was removed from the sepharose by boiling in 40 µl of SDS-PAGE sample buffer and subsequently separated by PAGE and visualised by autoradiography as described above.

2.4.8. cDNA synthesis.

1. cDNA to be used for a cDNA library was made with a cDNA Synthesis Plus kit (Amersham Int.) using the protocol and reagents supplied by the manufacturer and as described briefly below.

The first cDNA strand was synthesised from 1 µg mRNA isolated using mAP (as described in section 2.4.3) using 20 units of reverse transcriptase and primed with oligo-dT at 42°C for 40 mins. using the buffers supplied and incorporating 0.5 µl of α^{32}P dCTP (3000 Ci mMol^{-1}). The second strand was synthesised by adding 0.8 units of RNase H, 23 units of DNA polymerase I and 5 µl α^{32}P dCTP to the first round products and incubating in the supplied buffer for 60 mins. at 12°C, followed by 60 mins. at 22°C and then 10 mins. at 70°C. Any gaps were "filled in" by adding 2 units of DNA polymerase I and incubating for 10 mins. at 37°C. The reaction was stopped by adding 4 µl 0.25 M EDTA, pH. 8, the cDNA extracted with phenol:chloroform and precipitated overnight at -20 °C by adding 0.3 vols. Na. acetate pH. 5.2 and 2

vols. of ethanol. The cDNA was recovered by centrifuging at 13,000 g in a microfuge, washed gently in 70% ethanol, dried and resuspended in 10 μl dH_2O.

2. Single strand cDNA for use in PCR experiments was synthesised using a modification of the method described by Frohman and Martin (1989) using the following primer
5' GACTACGTTAGCATCTAGAATTCTCGAG-$(DT)_{17}$ 3'.

1 μg mRNA in 15.75 μl H_2O, prepared using the poly (A) Quik kit as described in section 2.4.3 was heated to 65°C for 3 mins., cooled on ice and then added to 2 μl 10X reverse transcriptase buffer (0.5 M Tris.HCl, pH 8.15, 60 mM $MgCl_2$, 40 mM KCl, 1 mM dithiothreitol, 1.5 mM each dNTP [ultra-pure, Pharmacia], 1 μl RNasin [Amersham Int.] and 0.5 μl primer [1 $\mu g ml^{-1}$]. 10 units of murine moloney reverse transcriptase (Pharmacia) was then added and the mixture incubated for 2 hrs at 41 °C. Following incubation the mixture was diluted to 1 ml with TE (10mM Tris.HCl, pH 7.5, 1 mM EDTA) and stored at 4°C.

2.4.9. Preparation of lambda plating cells.

A single bacterial colony was inoculated into L-broth (10g peptone, 5g NaCl and yeast extract per litre) supplemented with 0.2 % (w/v) maltose and 10 mM $MgSO_4$ and incubated with vigorous shaking (200 rpm) overnight at 37°C. 1 ml of culture was then transfered to 100 ml of L-broth (plus maltose and $MgSO_4$) and

further incubated until the A_{600} was in the range 0.7-0.9. The cells were pelleted by centrifugation at 1,000g for 10 mins. and then gently resuspended in 0.4 vols. of cold 10 mM $MgSO_4$. The cells were stored at 4°C and used for up to one week.

2.4.10. Plating of bacteriophage lamda.

Phage were plated onto either 9 (≈ 2000-3000 phage) or 15 cm (8,000-10,000 phage) petri dishes. Aliquots of phage in 100 µl SM were incubated for 30 mins. with 100 µl (9 cm plates) or 500 µl (15 cm plates) of plating cells at 37°C in a sterile 15 ml test tube. After incubation 3 ml (9cm) or 7 ml (15 cm) molten agarose (0.7%) containing 10 mM $MgSO_4$ cooled to 47°C was added to each tube, vortexed, poured on to the relevant sized plate containing set L-agar (pre-warmed to 37°C) and swirled to to form an even covering of the agar. The plates were left to harden for 10 mins., inverted and then incubated overnight at 37°C.

2.4.11. cDNA library construction.

The vector used for the construction of the cDNA library was the lamda expression vector gt11 (Young and Davis,1983). This contains the lacZ gene of E.coli with a unique EcoR1 site near the 3' end of its lacZ gene, into which foreign DNA can be inserted. The resulting gene fusion enables the cloned sequence to be expressed as a fusion peptide. In cells containing the lac repressor expression is inhibited until the repressor is

inactivated by the addition of the inducer IPTG. E.coli strain Y1090 was used as the host for the cDNA library as it allows lytic growth and plaque formation. In the presence of both IPTG and the chromogenic substrate X-gal, a blue colour is formed in parental phage plaques while recombinant phage remain white. This permits the proportion of recombinants in a cDNA library to be determined.

cDNA was cloned into lambda gt11 using the cDNA cloning system (Amersham Int.) using the protocol and buffers supplied by the manufacturer as described briefly below.

1 μg of cDNA synthesised as described in section 2.4.8 was methylated using 20 units of Eco R1 methylase to protect internal EcoR 1 sites. Eco R1 linkers were then added to the cDNA by ligation with T4 DNA ligase, the linkers digested with Eco R1 to generate EcoR 1 cohesive ends, the cDNA separated from excess linkers by size exclusion chromatography and then ethanol precipitated. Two ligation reactions were performed by ligating 50 and 100 ng of linkered cDNA to 0.5 μg gt11 Eco R1 phosphatased arms with 2.5 units of T4 DNA ligase for 20 hrs at 15°C and then the entire contents of each ligation reaction packaged using the extracts supplied. Unlike the genomic library, the cDNA library was not amplified as amplification of gt11 libraries can cause a reduction in the abundance of poorly growing recombinants.

2.4.12. Immunoscreening the cDNA library.

The cDNA library was screened with antibodies vs Pr1 using the Super Screen system (Amersham). In this system a protein imprint of the expression library is prepared on nitrocellulose filters and this is then screened with specific antibodies. Positive plaques are then identified using a horseradish peroxidase-labelled second antibody (anti-rabbit IgG). The immunoscreening system was used according to the protocol supplied and is described briefly below.

Nitrocellulose filters (Hybond-C, Amersham Int.) were first impregnated with IPTG by wetting the filters in a solution of 10 mM IPTG and then drying on paper towels. The filters were then laid on to the plated library (5000 plaques per 90 mm plate) and the plates incubated for a further 3 hrs at 42°C to induce expression of the fusion peptide. The filters were then lifted off the plates, washed 3x in TBS (10 mM Tris.HCl, pH 7.4, 150 mM $MgSO_4$) and then blocked by incubating overnight in a 2% solution of BSA in TBS at 4°C. The filters were incubated for 1 hr with anti-Pr1 antibody (1:50 in TBS plus 0.2% BSA), washed 4x in TBST (TBS plus 0.05% Tween 20) and then incubated in the peroxidase linked anti-rabbit IgG supplied. The substrate colour was then developed by incubating the filters in 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide as described by the manufacturer.

2.4.13. Fungal DNA extraction.

The DNA extraction method was essentially as described by Raeder and Broda (1985): Mycelium was prepared by inoculating 100 ml of complete medium in 250 ml conical flasks with 10^6 conidia. The cultures were incubated at 100 rpm for 48 hrs at 27°C. Mycelium was collected over a Buchner funnel, washed with 20 mM EDTA pH 8, ground to a fine powder under liquid nitrogen and then lyophilised overnight. The dried powder was resuspended in Tris-HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS and gently shaken for 30 mins. Phenol/chloroform (7:3), equilibrated with suspension buffer, was added and the suspension shaken for a further 30 mins. The suspension was centrifuged at 12000g for 1 hr at 4°C. The aqueous phase was then extracted once with phenol/chloroform and once with chloroform:isoamyl alcohol (24:1). RNase was added to the supernatant at 0.1 mg/ml and incubated at 37°C for 30 mins. DNA was precipitated with 0.1 vols. of sodium acetate pH 5.2 and 2 vols. of ethanol overnight at -20°C. DNA was recovered by centrifugation at 12,000g for 20 mins. The pellet was washed gently with 70% ethanol, dried and resuspended in dH_2o.

2.4.14. Genomic library construction

The vector chosen for the construction of the genomic library was the lambda replacement vector EMBL3 (Frischauf, et al., 1983). This vector can accommodate a large amount of foreign DNA (up to

23 kb) and has a polylinker sequence flanking the middle fragment. E.coli strains LE392 and P2392 were used as hosts for plating and titering the genomic library. These strains are similar except that P2392 has a P2 prophage inserted into the genome which only allows growth of recombinant phage without the central 'stuffer' fragment.

1. Preparation of random 15-20 kb DNA fragments:

Random DNA fragments were generated by partial digestion of high molecular weight DNA with the restriction endonuclease Mbo 1. A pilot experiment was carried out to determine the optimum conditions to generate 15-20 kb fragments. The enzyme was used at concentrations of between 2 and 1/64 units per μg of DNA in a restriction digest for 1 hr at 37°C.

Approximately 150 μg of DNA in Mbo 1 buffer (supplied by the manufacturer) was mixed with 2.5 units of Mbo 1 and incubated at 37°C. Aliquots of 50 μg DNA were removed at 30, 60 and 90 min. intervals.

2. Size fractionation of DNA fragments:

The DNA fragments from the Mbo 1 digestion were fractionated on a glycerol gradient. 150 μg of digested DNA was loaded on to 10-30% discontinuous glycerol gradient formed with 10, 15, 20, 25 and 30% glycerol solutions in 10 mM Tris-HCl pH 8, 200 mM

NaCl, 20 mM EDTA. The gradient was centrifuged at 20,000g for 16 hrs at 4°C. Fractions of 500 µl were taken and aliquots examined by agarose gel electrophoresis. Fractions containing 15-20 kb fragments were pooled and the DNA precipitated with 0.1 vols. 3 M sodium acetate pH 5.2 and 2 vols. ethanol overnight at -20°C. The DNA was recovered by centrifugation at 12,000g for 20 mins. at 4°C. The pellet was washed gently with 70% ethanol, dried and resuspended in dH_2O.

3. Ligation and packaging:

0.4 µg of size fractionated Bam H1 digested genomic DNA was ligated to 1 µg Bam H1 pre-digested EMBL3 arms (Stratagene) with 3 Weiss units T4 DNA ligase (Northumbria Biologicals Ltd.) and 1 mm ATP in a total volume of 5 µl ligase buffer (50 mm Tris.HCl, pH8, 7 mm $MgCl_2$, 1 mm dithiothreitol) for 1 hr at room temperature and then overnight at 4°C. 1 µl of the ligation reaction was then packaged using the Gigapack Plus packaging extract (Stratagene) according to the protocol supplied by the manufacturer.

4. Amplification of the library:

The entire contents of the packaging extract was plated with P2392 plating cells to select for recombinant phage. The genomic library was amplified by flooding each plate lysate with 5 ml of SM buffer (0.58g NaCl, 0.2g $MgSO_4.7H_2O$, 5 ml 1 M Tris. HCl pH75,

0.5 ml 2% gelatin, H_2O to 100 ml) followed by gentle shaking overnight at 4°C. The phage suspension was removed and chloroform added to 5% and the suspension gently shaken for 15 mins. at room temperature. The phage suspension was centrifuged at 5,000g for 5 mins. and the supernatant made to 0.3% chloroform. The titre of the amplified library was then determined by plating with P2392 cells.

2.4.15. Immobilisation of lambda plaques on nitrocellulose filters.

Overnight phage plates were cooled at 4°C for 1 hr to allow the top agarose to harden. A circular nitrocellulose filter (Schleicher and Schuell BA85) was then placed onto the surface of the top agarose and the orientation of filter marked by stabbing through the filter in 3 assymetric locations with a needle previously dipped in bromophenol blue DNA loading buffer. After 1 min. the filter was removed, immersed DNA side up in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 1 min., then in neutralising solution (1.5 M NaCl, 0.5 M Tris. HCl [pH7.5]) for 5 mins., rinsed in 2x SSC and placed DNA side up on a piece of whatman 3MM paper. A replica filter was similarly made, but left on the top agarose for 2 mins. and marked in the same positions as the original filter. After the filters were dry the DNA was fixed to the nitrocellulose by baking for 2 hrs at 80°C. The filters were then stored at 4°C prior to hybridisation to ^{32}P-labelled oligonucleotides.

2.4.16. Southern blotting.

The method used to transfer and immobilise DNA from agarose gels on to nylon membranes was a modification of the method described by Southern (1975): After electrophoresis the gels were depurinated for 30 mins. in 0.25% HCl, denatured for 2x 20 mins. in denaturing bufer (0.5 M NaOH, 1.5 M NaCl) and then neutralised in neutralising buffer (0.5 M Tris.HCl, pH7.5, 1.5 M NaOH) for 2x 20 mins. The DNA was transferred overnight to nylon membranes (Hybond-N, Amersham Int.) by placing the washed gel on to 5 pieces of whatman 3MM paper soaked in neutralising buffer and placing a stack of paper towels with a one kilogram weight on the gel. After transfer the gel was washed in 2x SSC, wrapped in saran wrap and the DNA fixed to the membrane by exposure to UV light for 5 mins.

2.4.17. Oligonucleotides; synthesis and purification.

Oligonucleotides were purchased from the Dept. of Biochemistry, Bath University, where they were synthesised on an Applied Biosystems model 381A synthesiser using β-cyanoethyl phosphoramidite nucleoside derivatives.

After synthesis the oligonucleotides were removed from the Controlled Pore Glass Column by passing 5X 0.2 ml aliquots of concentrated ammonia through the column; the solution was then incubated overnight at 55°C to remove protecting groups on the

exocyclic amines of the bases. The oligonucleotides were then dried under vacuum, after resuspension in 1 ml of dH_2O the oligonucleotides were precipitated with 0.1 ml Na acetate pH 5.2 and 3 ml ethanol overnight at -20°C. The oligonucleotides were recovered by centrifuging at 13,000g in a microfuge, dried and resuspended in 1 ml dH_2O.

The quality of the oligonucleotides was assessed by electrophoresis through a denaturing polyacrylamide gel as described by Sambrook et al. (1989). 10 μg DNA was mixed with an equal volume of formamide, incubated at 55°C for 5 mins. and then loaded on to a 19% polyacrylamide gel (31.5g urea, 37.5 ml 20% acrylamide, 15 ml 5x TBE) and run at 800V. The gel was monitored by loading standard bromophenol blue loading buffer into a single well. After electrophoresis the gel was placed on a fluorescent thin-layer chromatography plate and examined under long wave UV light. Oligonucleotides which did not appear as individual homogeneous bands of the correct size were cut from the gel and purified as follows. The gel slice was crushed in 1 ml of 0.1% SDS, 0.5 M ammonium acetate, 10 mM magnesium acetate and incubated for 12 hrs in an orbital shaker (150 rpm) at 37°C. The solution was centrifuged at 12,000g for 5 mins. and then passed through a 0.45-micron filter (millipore). The oligonucleotide was isolated from the solution by reverse phase chromatography on a Sep-Pak C_{18} column. The column was prepared by pushing 10 ml HPLC grade acetonitrile through the column followed by 10 ml dH_2O and 2 ml 10 mM ammonium acetate. The

oligonucleotide solution was then added to the column followed by 3x 10 ml washes of dH_2O. The oligonucleotide was recovered from the column by passing 3x 1 ml 60% methanol through the column. The solution was then dried under vacuum and the purified oligonucleotide resuspended in 200 μl of dH_2O.

2.4.18. Labelling of oligonucleotides.

Synthetic oligonucleotides were radioactively labelled using [γ-^{32}P]ATP and bacteriophage T4 polynucleotide kinase as described by Sambrook et al. (1989).

Approximately 10 pmoles of oligonucleotide were mixed with 2 μl 10x kinase buffer (0.5 M Tris. HCl, pH 7.6, 0.1 M $MgCl_2$, 50 mM dithiothreitol, 1 mM spermidine, 1 mM EDTA), 5 μl [γ-^{32}P]ATP (6000 Ci mmole^{-1}) and H_2O to 20 μl. Then 8 units ($\approx$1 μl) T4 polynucleotide kinase was added, the reaction incubated for 45 mins. at 37°C and then the enzyme inactivated by incubating the reaction at 68°C for 10 mins.

The efficiency of transfer of ^{32}P to the oligonucleotide was then determined by spotting 0.5 μl of the reaction on to a strip of cellulose impregnated with polyethyleneimine (Polygram CEL 300PEI; Brinkman) and the strip then placed in 0.5 M ammonium bicarbonate until the solvent front migrated about 3 inches. The cellulose was then wrapped in saran wrap and autoradiographed for 5 mins. Since the oligonucleotides remain at the origin and free

^{32}P migrates behind the solvent front, the approximate labelling efficiency can be determined. Labelling usually resulted in ca. 50% of the phosphate being transferred from the [γ-^{32}P]ATP to the oligonucleotide.

2.4.19. Hybridisations.

Southern blots were prehybridised in tubes in a rotary hybridisation oven (Hybaid) for 1-2 hours in 10 ml of 5x SSC, 50 mM Na phosphate pH 7, 10x Denhardt's solution and 7% SDS at 37°C. The probe was added directly to the prehybridisation solution. After hybridisation overnight the filters were washed with 5x SSC, 1% SDS for 15 mins. at room temperature followed by two washes at various temperatures as described in the results.

Phage plaque lifts were hybridised to radioactive oligonucleotides in 15 cm^2 sandwhich boxes in an orbital incubator with gentle shaking. Hybridisation solutions and conditions varied as described in the results.

After stringency washes, the filters and blots were then blotted dry and wrapped in Saran wrap prior to autoradiography using Fuji X-ray film and cassettes fitted with intensifying screens at -70°C for 1-3 days.

2.4.20. Picking bacteriophage lambda plaques.

Positively hybridising plaques were picked by stabbing through the plaque with a pasteur pipette fitted with a rubber bulb into the underlying agar. The plaque and the agar were removed by applying mild suction, placed in 1 ml of SM containing 1 drop of chloroform and incubated for 1-2 hrs at room temperature or 4°C overnight.

Individual plaques were also isolated by touching the plaque with a sterile toothpick, touching the end of the pick on to a plate containing L-agar overlaid with set agarose and plating cells as described above and incubating overnight at 37°C.

2.4.21. Mini-preparations of lambda DNA.

A single plaque was suspended in 200 µl of SM and 100 µl of this added to 50 µl of plating cells and incubated at 37°C for 20 mins. The cells were added to 10 ml of L-broth with 10 mM $MgSO_4$ and incubated with shaking at 37°C until lysis. After lysis 100 µl of chloroform was added and the cells centrifuged at 5000g for 10 mins. To 0.8 ml of lysate, DNase was added to 10 µg/ml and incubated at room temperature for 15 mins. Then, 200 µl 0.3 M Tris-HCl pH 9, 0.15 M EDTA, 1.5% SDS was added. After gentle mixing, the lysate was incubated for 15 mins. at 70°C then quenched on ice. 135 µl of 8 M potassium acetate was added and the lysate incubated for a further 15 mins. at 4°C. Cell debris

was removed by centrifugation in a microfuge for 2 mins and the DNA then precipitated by the addition of 0.6 vols. of isopropanol. The DNA was recovered by centrifuging in a microfuge for 5 mins. The pellet was washed gently with 70% ethanol, dried and resuspended in dH_2O.

2.4.22. Polymerase chain reactions (PCR).

All PCR experiments were performed using a Hybaid thermal reactor (HB-TR1) with temperature controlled using a tube thermocouple. The thermostable DNA polymerase used in all experiments was "Replinase" (Du Pont) using the 20x reaction buffer (1M Tris-HCl, pH 9, 400 mM ammonium sulphate, 30 mM magnesium chloride) supplied by the manufacturer.

2.4.23. DNA sequencing.

PCR products were sequenced directly by the chain termination method of Sanger et al. (1977) with Sequenase (New England Biochemicals) using a modification of the manufacturers protocol.

The DNA was annealed to the primer by mixing 7 μl denatured DNA solution, 2 μl sequencing buffer and 5 pmoles primer, incubating at 65°C for 2 mins. and then chilling on ice. The reaction was then labelled by adding to the 10 μl annealing mixture 1 μl dithiothreitol, 2 μl dNTP mix (0.5 mM each of dATP, dGTP, dTTP) 0.5 μl ^{32}P-dCTP (3000 Ci mmol^{-1}) and 2 μl Sequenase Version 2

(diluted 1:8 in dilution buffer). The reactions were vortexed and immediately chilled on ice. The reaction was terminated by transferring 3.5 µl of the labelling reaction to 2.5 µl of each ddTP, incubating for 5 mins. at 37°C and then stopped by the addition of 4 µl of stop buffer. The samples were then heated at 75°C for 2 mins., then 3 µl of each termination reaction loaded on to a 6% denaturing polyacrylamide gel (6% acrylamide, 7 M urea in 1x TBE) and run in 1X TBE at 1.6 kV until the bromophenol blue reached the end when a further 3 µl of each sample was loaded on to the gel and run until the dye front had reached the end. The top plate was then removed, the gel fixed in 5% methanol, 5% acetic acid for 15 mins. at room temperature, washed in dH_2O, dried on the bottom plate with a hair drier and then autoradiographed overnight with Fuji X-ray film at room temperature.

The top "lugged" plate was washed in ethanol and siliconised using repelcote (BDH) prior to pouring the gel. The bottom plate was washed in ethanol, coated in a solution of 15 ml ethanol, 450 µl 10% acetic acid and 75 µl 2-methacryloxypropyltrinethoxy-silane and rinsed in dH_2O prior to use.

3. PROTEASE REGULATION

3.1. RESULTS

The need to obtain large quantities of protease for purification and to establish culture conditions in which fungal cells may contain a high proportion of protease specific mRNA for cDNA cloning required a study of protease regulation in *M. anisopliae*.

Previous work has shown that Pr1 and Pr2 production is controlled by carbon and nitrogen derepression (St. Leger et al., 1988b). However, recent observations by the author and some published data (Kucera, 1981; St. Leger et al., 1988b, 1991b) indicates that protease production is higher when the fungus is grown on insect cuticle (but not on other mono or polymeric carbon sources) than is achieved by derepression alone. This suggests that a component of cuticle may induce protease production. A series of experiments was performed to investigate this possibility for Pr1 and Pr2 production.

3.1.1. Derepression of protease production.

To establish a fungal biomass, mycelium (≈ 300 mg dry wt.) was grown for 3 days in complete medium (CM) and the effect of starvation on protease production examined by transferring mycelia to basal salts medium lacking either carbon (C), nitrogen

(N), SO_4 (S) or both carbon and nitrogen (-C-N); protease production was assayed after 24 hrs.

Neither Pr1 nor Pr2 was produced in media lacking either C, N or S. In -C-N media low levels of Pr1 (45.6 ± 9.3 nmoles NA ml^{-1} min^{-1}) and Pr2 (18.1 ± 4.5 nmoles NA $ml^{-1}min^{-1}$) were detected after 24 hrs confirming that protease production can occur by combined C and N derepression alone.

3.1.2. Effect of different carbon and nitrogen sources on protease production.

Mycelium from 3-day CM cultures was incubated for 24 hrs in basal salts medium lacking C and N; after this period of starvation different C and N sources (1% w/v) were added and protease activity assayed for up to 24 hrs (Table 2).

Addition of elastin, collagen, cellulose or xylan did not enhance Pr1 production and the addition of the soluble proteins BSA and gelatin completely repressed Pr1 production. However, addition of locust cuticle enhanced Pr1 production to a level ca. 10 times that of control (-C-N) flasks after 16 hrs (Fig. 1). Thus, the enhancing effect of certain insoluble polymers (eg. cellulose) as described by St. Leger et al. (1988, 1991) was not evident in this study, but some slight enhancing effect was seen with elastin 8 hrs after adding the protein, but Pr1 levels subsequently fell to that of the control flasks. The insoluble

Table 2

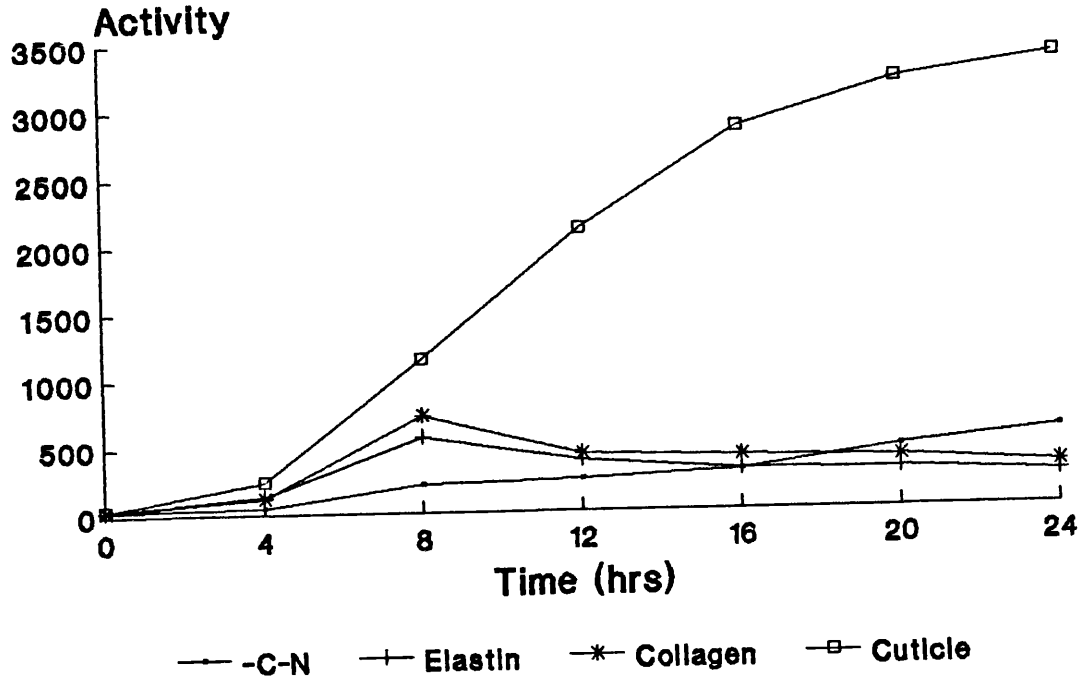
Protease production by derepressed mycelium of *Metarhizium anisopliae* on a range of polymeric substrates

	Enzyme activity[1]	
C/N source (1%)	Pr1	Pr2
Control (-C-N)	293.3 ± 15.4	33.6 ± 3.5
Cuticle	2841.6 ± 75.9	461.1 ± 48.4
Elastin	298 ± 10.7	259.7 ± 25.8
Collagen	396.2 ± 21.3	297.3 ± 27.9
BSA	0	396.1 ± 42.6
Gelatin	0	247.4 ± 19.2
Cellulose	89.4 ± 27.8	29.5 ± 6.7
Xylan	270.5 ± 11.7	28.7 ± 7.1

[1] Mean activity ± SD from 3 replicates 16 hrs after addition of C/N source to mycelium starved of C and N for 24 hrs. Enzyme activity expressed as nmolNA $ml^{-1}min^{-1}$ liberated from Suc-$(Ala)_2$-Pro-Phe-NA (Pr1) and Bz-Phe-Val-Arg-NA (Pr2). The results are representative of three similar experiments.

Figure 1

Effect of polymeric substrates on Pr1 production by derepressed mycelium of Metarhizium anisopliae



Mycelium from 3-day CM cultures was starved of C and N for 24 hrs in basal salts before the addition of different polymers (1% w/v). Pr1 production was assayed at different times for 24 hrs. Activity is expressed as nmol NA $ml^{-1}min^{-1}$ liberated from Suc-$(Ala)_2$-Pro-Phe-NA. Pr1 production by mycelium after the addition of cellulose and xylan was similar to that of -C-N (not shown). No Pr1 was produced in cultures containing BSA or gelatin. Enzyme activities represent the mean of three replicates; the experiment was repeated three times with similar results.

protein collagen also slightly enhanced Pr1 production to levels twice those of controls but 7-fold less than cuticle grown cultures (Fig.1)

The time course of Pr2 production is shown in Fig. 2 and the effect of different carbon sources in Table 2. Production of Pr2 was enhanced by the addition of cuticle, elastin, collagen, gelatin and BSA but not cellulose or xylan. After 12 hrs growth on cuticle or BSA Pr2 activity was ca. 12.5x that seen in -C-N flasks and after 24 hrs growth on cuticle Pr2 levels was ca. 18-fold greater than in controls.

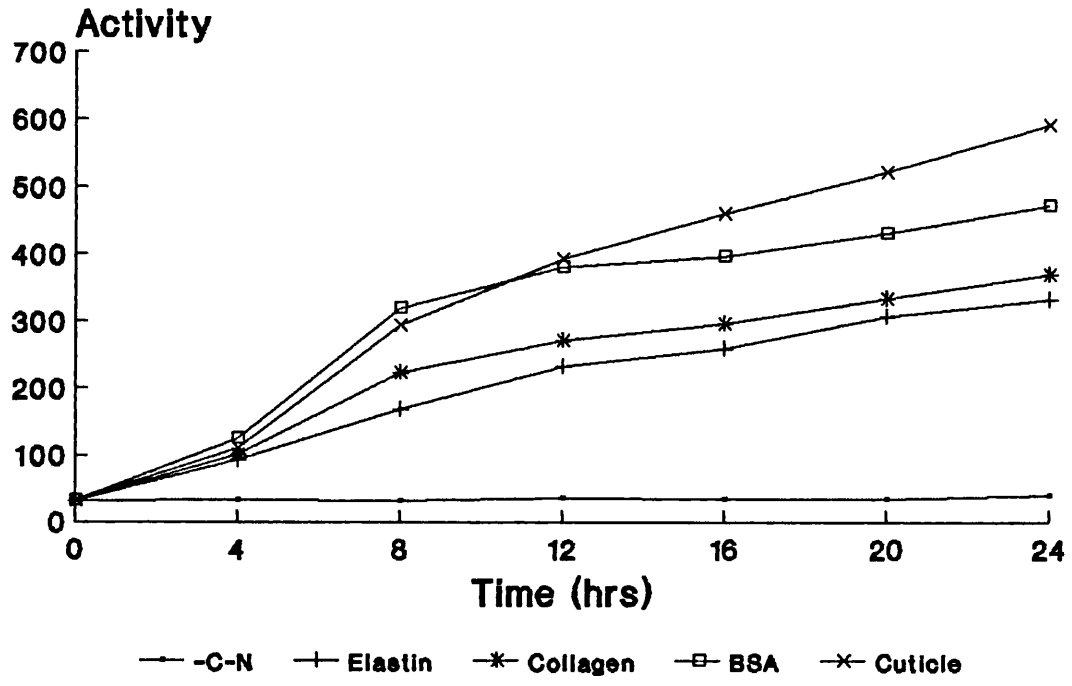
These results suggest that production of these two proteases is not controlled by derepression alone. Pr1 appears to be specifically induced by insect cuticle whereas Pr2 appears to be induced by any protein, whether soluble or insoluble; this suggests that Pr2 is less susceptible to catabolite repression than Pr1.

3.1.3. Effect of nutrient limitation on protease production by protein induced cultures.

Studies on protease regulation in fungi have revealed that different regulatory mechanisms exist between species. Most Aspergillus species synthesise protease upon limitation of C, N or S (Cohen, 1981). Other fungi such as N. crassa (Drucker, 1972, 1975; Cohen et al., 1975; Cohen and Drucker, 1977) and some

Figure 2

Effect of polymeric substrates on Pr2 production by derepressed mycelium of Metarhizium anisopliae



Mycelium from 3-day CM cultures was starved of C and N for 24 hrs in basal salts before the addition of different polymers (1% w/v). Pr2 production was assayed at different times for 24 hrs. Activity is expressed as nmol NA $ml^{-1}min^{-1}$ liberated from Bz-Phe-Val-Arg-NA. Pr2 production by mycelium after the addition of cellulose and xylan was similar to that of -C-N (not shown). Enzyme activities represent the mean of three replicates; the experiment was repeated three times with similar results.

Candida species (Rembold et al., 1968; Crandall and Edwards, 1987) require both nutrient limitation and an inducing protein to effect protease production. The effect of adding protein (BSA) and cuticle to mycelia starved of C, N, or S was investigated.

Mycelium from 3-day CM cultures was transferred to basal salts deficient in one of C, N or S plus or minus either BSA or cuticle. The medium used was buffered basal salts plus trace elements (see section 2.2.1) plus sucrose (1% w/v) or NH_4Cl (0.2% w/v). Medium deficient in S contained $MgCl_2$ instead of $MgSO_4.7H_2O$; similarly chlorides were substituted for the sulphates in the trace elements. Protease activity was measured 8 and 16 hrs after transfer.

Pr1 was repressed in all media containing N but deficient in C (Table 3). No Pr1 was produced in media lacking N (but containing C) unless cuticle was present in which case Pr1 levels were approximately 30% of those produced in -C-N media plus cuticle. No Pr1 was produced in media lacking only S.

High levels of Pr2 were detected in -N and -C-N media plus BSA or cuticle (Table 4). Only trace levels of Pr2 were detected with N or C derepression alone or in -C plus cuticle medium after 16 hrs, but not after 8 hrs incubation. Basal levels of both proteases were produced in media lacking both C and N.

It is apparent that Pr1 and Pr2 are regulated differently; Pr2

Table 3

Effect of carbon, nitrogen or sulphur repression on Pr1 production by Metarhizium anisopliae.

	Enzyme activity[1]	
C/N source (1%)	8 hrs	16 hrs
-C-N + Cuticle	159.1 ± 30.9	818.2 ± 66.3
-C + Cuticle	0	0
-N + Cuticle	56.8 ± 15.9	254.5 ± 27.2
-S + Cuticle	0	0
-C-N + BSA	0	0
-C + BSA	0	0
-N + BSA	0	0
-S + BSA	0	0
-C-N	9.1 ± 4.5	40.9 ± 6.3
-C	0	0
-N	0	0
-S	0	0

[1]Mean activity ± SD from 3 replicates 8 and 16 hrs after the transfer of mycelia to basal salts deficient in either C, N or S plus Cuticle or BSA (1% w/v). Enzyme activity expressed as nmolNA $ml^{-1}min^{-1}$ liberated from Suc-$(Ala)_2$-Pro-Phe-NA. The results are representative of three similar experiments.

Table 4

Effect of carbon, nitrogen or sulphur repression on Pr2 production by Metarhizium anisopliae.

	Enzyme activity[1]	
C/N source (1%)	8 hrs	16 hrs
-C-N + Cuticle	241.9 ± 16.2	472.4 ± 39.6
-C + Cuticle	0	15.9 ± 2.3
-N + Cuticle	229.6 ± 11.4	436.4 ± 19.7
-S + Cuticle	0	0
-C-N + BSA	212.3 ± 19.8	396.1 ± 27.5
-C + BSA	0	0
-N + BSA	192.4 ± 11.7	403.2 ± 42.7
-S + BSA	0	0
-C-N	27.2 ± 5.2	29.5 ± 7.8
-C	0	13.6 ± 4.5
-N	0	20.45 ± 9.1
-S	0	0

[1]Mean activity ± SD from 3 replicates 8 and 16 hrs after the transfer of mycelia to basal salts deficient in either C, N or S plus Cuticle or BSA (1% w/v). Enzyme activity expressed as nmolNA $ml^{-1}min^{-1}$ liberated from Bz-Phe-Val-Arg-NA. The experiment was repeated three times with similar results.

is produced under conditions where protein acts as the sole N source whereas maximal synthesis of Pr1 is achieved upon limitation of C and N in the presence of insect cuticle. These data further suggest the possibility that Pr1 and Pr2 are induced specifically by cuticle and non-specifically by proteins respectively.

3.1.4. Effect of protein substrate concentration on protease production.

Earlier experiments have shown that Pr1 is synthesised by M. anisopliae after addition of cuticle (1% w/v) to mycelia starved of C and N. Pr2 is produced in the presence of either cuticle, BSA, collagen or gelatin. Of the proteins tested, the addition of BSA resulted in the highest levels of Pr2 activity, but it also completely repressed Pr1 production. It is possible that addition of cuticle or BSA at a concentration of 1% may not result in optimal protease production, and the catabolite repression of Pr1 by BSA may not occur at lower BSA concentrations.

To examine this possibility cuticle or BSA was added to mycelia starved of C and N for 24 hrs (as previously described) at concentrations of 0.2, 0.5, 1 and 2%; protease activity was then measured 12 hrs after addition of the substrate (Table 5).

The levels of Pr1 and Pr2 activity were similar for cuticle concentrations of 0.5, 1 and 2%, but at 0.2% activities were 54

Table 5

Effect of substrate concentration on protease production in *Metarhizium anisopliae*

	Enzyme activity[1]	
C/N source	Pr1	Pr2
BSA 0.2%	200.1 ± 24.6	247.3 ± 11.5
BSA 0.5%	0	273.1 ± 17.9
BSA 1%	0	186.2 ± 16.4
BSA 2%	0	91.7 ± 13.4
Cuticle 0.2%	1009.4 ± 67.9	163.7 ± 9.2
Cuticle 0.5%	1863.8 ± 59.7	211.6 ± 15.9
Cuticle 1%	1839.1 ± 83.8	238.9 ± 10.8
Cuticle 2%	1786.4 ± 46.3	216.8 ± 19.7

[1] Mean activity ± SD from 3 replicates 12 hrs after addition of C/N source to mycelium starved of C and N for 24 hrs.
Enzyme activity expressed as nmolNA $ml^{-1}min^{-1}$ liberated from Suc-$(Ala)_2$-Pro-Phe-$_p$NA (Pr1) and Bz-Phe-Val-Arg-$_p$NA (Pr2). The results are representative of two similar experiments.

and 68% of maximal levels for Pr1 and Pr2 respectively. Maximum Pr2 production was achieved by the addition of 0.5% BSA , with the effects of catabolite repression evident at concentrations of 1% and above. Pr1 was completely repressed by BSA concentrations above 0.5%, but basal levels of activity were detected at 0.2%. The marked decline in production of both enzymes with increasing concentrations of BSA but not of cuticle suggests catabolite repression is effected by the soluble protein.

3.1.5. Effect of pre-induction starvation on protease production.

Experiments with N.crassa (Drucker, 1973; Cohen and Drucker 1977) have shown that a period of starvation for C and N prior to protease induction can alter the time of initiation and rate of enzyme synthesis, suggesting the existence of an intracellular pool of repressing metabolites. The possible existence of such a pool for M. anisopliae was investigated.

Mycelium from 3-day CM cultures was transferred to flasks containing basal salts or basal salts plus C (1% sucrose) or N (0.2% NH_4Cl) and incubated for various times between 1 and 24 hrs, before transfer to flasks containing basal salts plus cuticle; protease activity was measured after 12 hrs.

The type and duration of pre-induction starvation affected both Pr1 (Fig. 3a) and Pr2 (Fig. 3b) production. Maximal Pr1 production was achieved if the addition of cuticle was preceded

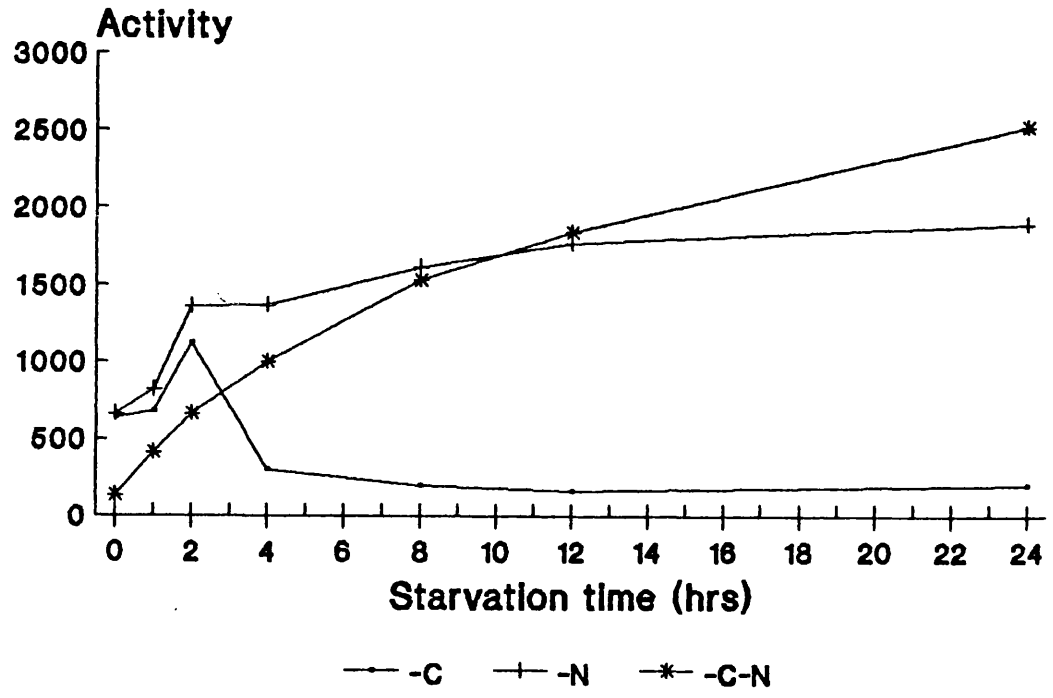
Figure 3

Effect of carbon and nitrogen starvation on subsequent protease production by Metarhizium anisopliae

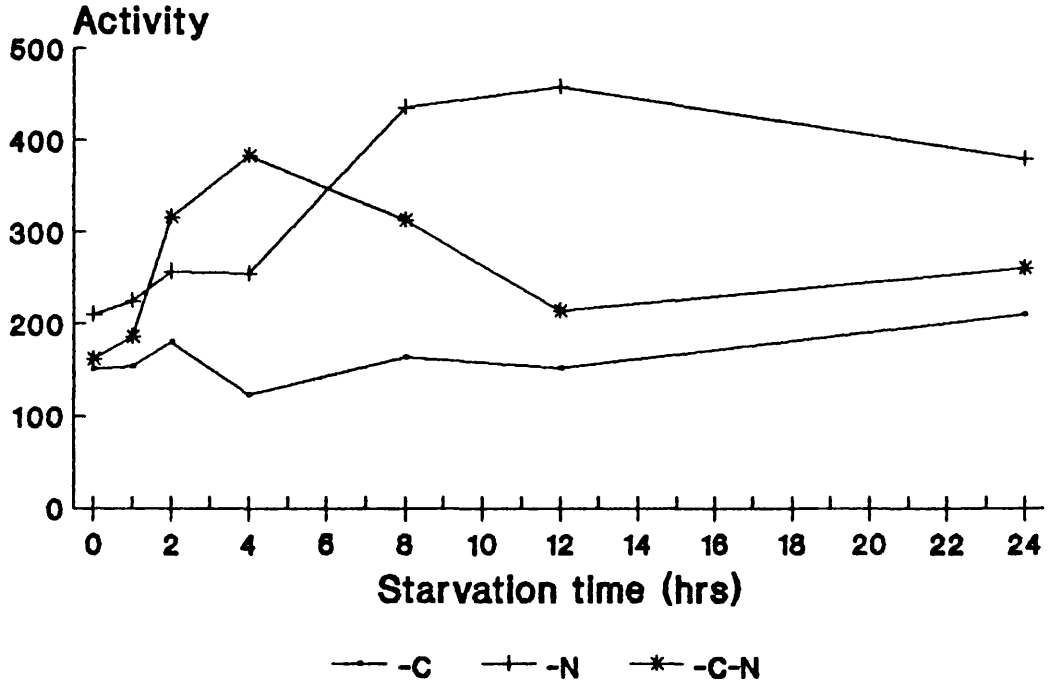
Mycelia from 3-day CM cultures was starved of C, N or C and N in buffered basal salts for various times before the addition of ground locust cuticle (1% w/v). Protease production was assayed 12 hrs after the addition of cuticle. Protease activity is expressed as nmolNA $ml^{-1}min^{-1}$ liberated from Suc-$(Ala)_2$-Pro-Phe-NA (Pr1) or Bz-Phe-Val-Arg-NA (Pr2). The results represent the mean of three replicates; the experiment was repeated three times with similar results.

Figure 3

Pr1



Pr2



by 2 hrs C starvation; longer periods of C starvation resulted in a fall in Pr1 activity to basal levels. Pr2 activity after 12 hrs growth appeared to be relatively unaffected by the duration of C starvation.

Mycelia pre-starved of N for 4 hrs produced 72% of maximum Pr1 activity, but maximum activity was achieved with a starvation period of 24 hrs. Maximum Pr2 activity occurred with a N starvation period of 12 hrs which fell to 83% of maximum with 24 hrs N starvation.

Pr1 levels from mycelia pre-starved of C and N continued to increase in relation to the duration of the starvation, with 50% of the maximal activity found after 12 hrs occurring after 6 hrs starvation. Maximum Pr2 activity resulted after 4 hrs C and N pre-starvation.

The requirement for a period of starvation of C and N before maximum protease activity is achieved suggests that pools of both C and N sufficient to repress protease production exist in the hyphae of M. anisopliae. The reduction in Pr1 production after periods of C starvation longer than 2 hrs may suggest that lower levels of C relative to N are available to be used in protease synthesis, but the fact that Pr2 production is relatively unaffected by periods of C starvation and that starvation for both C and N result in protease levels higher than those produced by C starvation alone would seem to contradict this possibility.

3.1.6. Estimation of fungal biomass in cultures with soluble and insoluble substrates.

Pr1 and Pr2 appear to be induced specifically by cuticle and non-specifically by protein respectively. A comparison of fungal growth in cultures is required to ensure that the ten-fold increase in Pr1 production on insect cuticle relative to that on other carbon sources is not due to an increase in fungal biomass. Separation of mycelium from insoluble substrates is impracticable, which precludes comparisons of mycelial dry weight. The assay methods available for measuring fungal biomass on solid substrates are chitin and ergosterol estimation (Matcham et al., 1984). Chitin is an integral part of insect cuticle structure and this necessitates the use of ergosterol for estimating biomass.

1) Development of an ergosterol assay as a measure of the biomass of M. anisopliae.

Ergosterol (24β-methylcholesta-5,7, trans 22-trien 3β-ol) is a unique fungal sterol (Weete, 1974) which can be used as a marker for estimating fungal biomass. Ergosterol has a pair of conjugated double bonds at carbons 5-6 and 7-8 which gives ergosterol a different u.v. absorption spectrum to other sterols. Measuring u.v. absorption at 282 nm enables ergosterol to be detected against a backround of other sterols which usually absorb very little light at wavelengths above 240 nm.

Ergosterol was separated and quantified by reverse phase HPLC using a Rainin C18 ODS2 column with detection at 282 nm. The HPLC system used is described in section 2.3.3. A mobile phase of 95% methanol and flow rate 2ml/min. gave good separation of the ergosterol peak from other extracted sterols; ergosterol standard (Fluka) gave a retention time of 10.5 mins. Ergosterol breaks down to give ergocalciferol (vitamin D) upon exposure to UV light (Tuksida, 1980), therefore all samples were stored in the dark. Ergocalciferol standard (Fluka) had a retention time of 7.5 mins. and could easily be distinguished from the ergosterol peak (Fig. 4). A calibration curve was produced using ergosterol standard (Fig. 5).

Ergosterol was extracted from mycelium as described in section 2.2.3 and resuspended in 20 ml methanol,of which 20 μl was analysed by HPLC. The chromatogram showed a peak with the same retention time as ergosterol standard, with no detectable interfering peaks and most other UV absorbing components did not bind to the column (Fig. 6). Spiking the sample with ergosterol standard did not give rise to any extra peaks indicating that the extracted sterol was ergosterol. This was further confirmed by collecting the presumed ergosterol peak and comparing its mass spectrogram with that of ergosterol standard (Fig. 7). The two spectra show similar fragmentation patterns, with fragments of molecular weights of 81, 363 and 379 Da being evident. The two spectra both indicate the presence of an unknown contaminant in the samples with a molecular weight of 621 Da. The two samples

Figure 4

Separation of ergosterol and ergocalciferol by reverse phase HPLC

Standard ergosterol (A) or ergocalciferol (B) were detected by absorption at 282 nm. Sterols were separated by HPLC using a Rainin C18 ODS2 reverse phase column with a mobile phase of 95% methanol run at 2 ml min^{-1}.

Figure 4

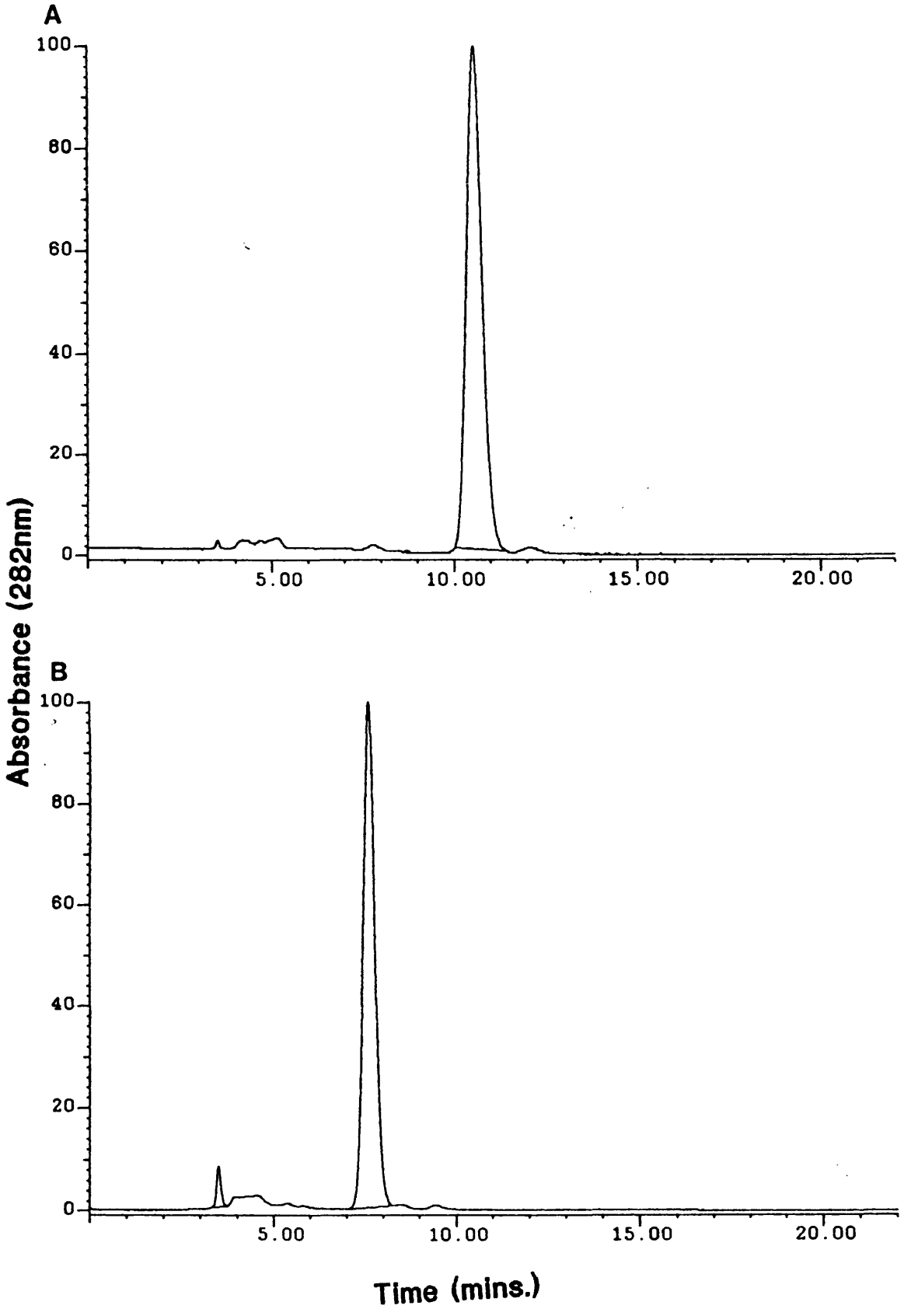
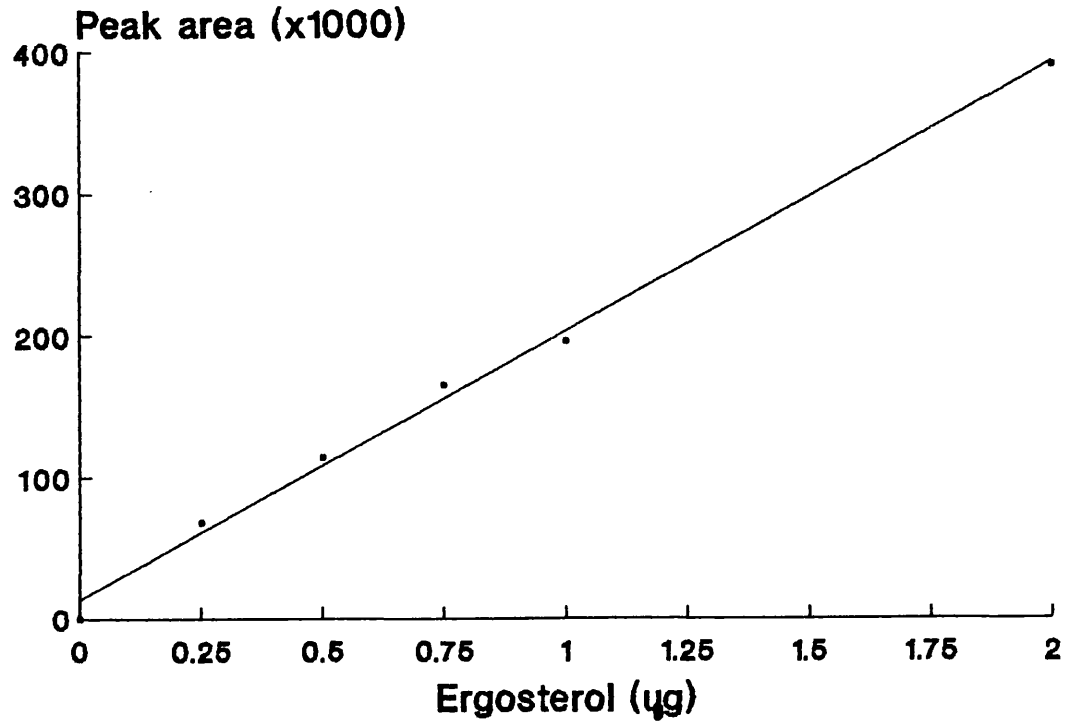

A
100
80
60
40
20
0
5.00
10.00
15.00
20.00
B
100
80
60
40
20
0
5.00
10.00
15.00
20.00
Absorbance (282nm)
Time (mins.)

Figure 5

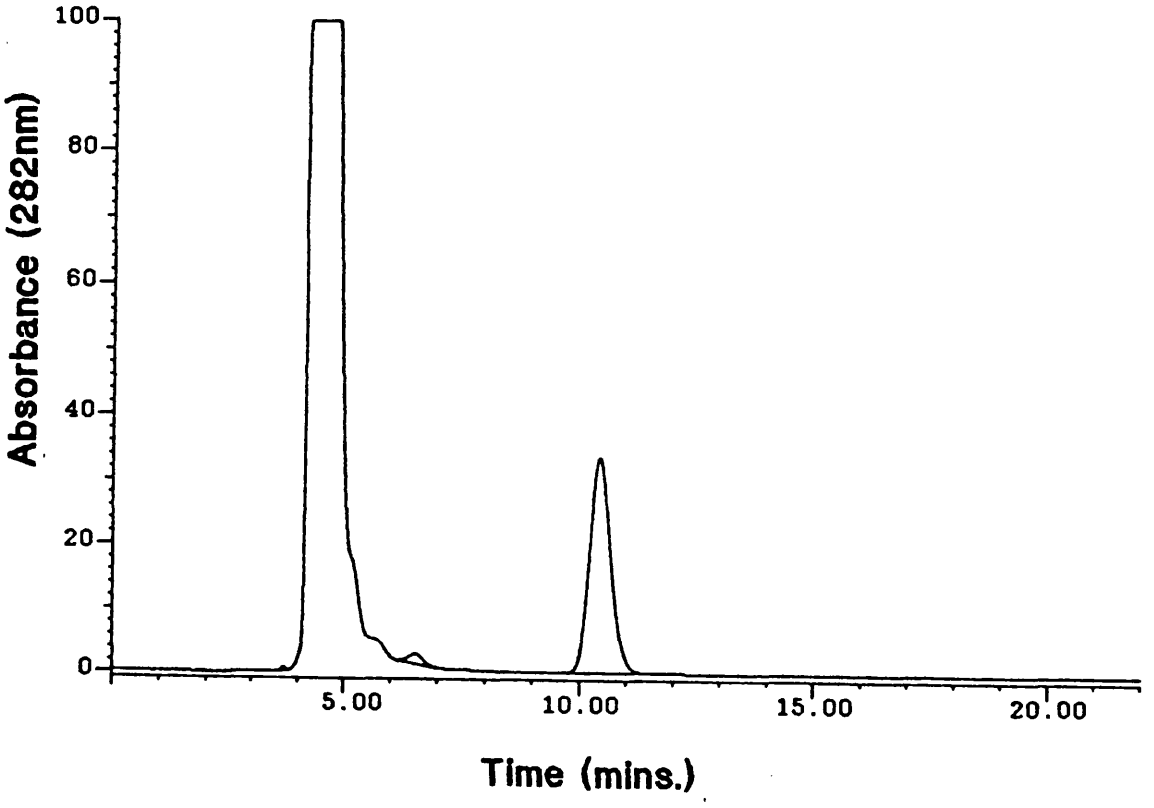
Calibration curve of ergosterol vs. peak area by HPLC



Ergosterol was analysed and detected as described for Fig. 4. The peak area was calculated (by integration) by the computer controlling the HPLC (see section 2.3.3).

Figure 6

Separation of ergosterol from other extractable sterols from mycelium of Metarhizium anisopliae



Sterols were extracted from mycelium from a 3-day CM culture as described in the text and analysed as described for Fig. 4. Ergosterol had a retention time of ca. 10.5 mins. with all other UV absorbing material failing to bind to the column.

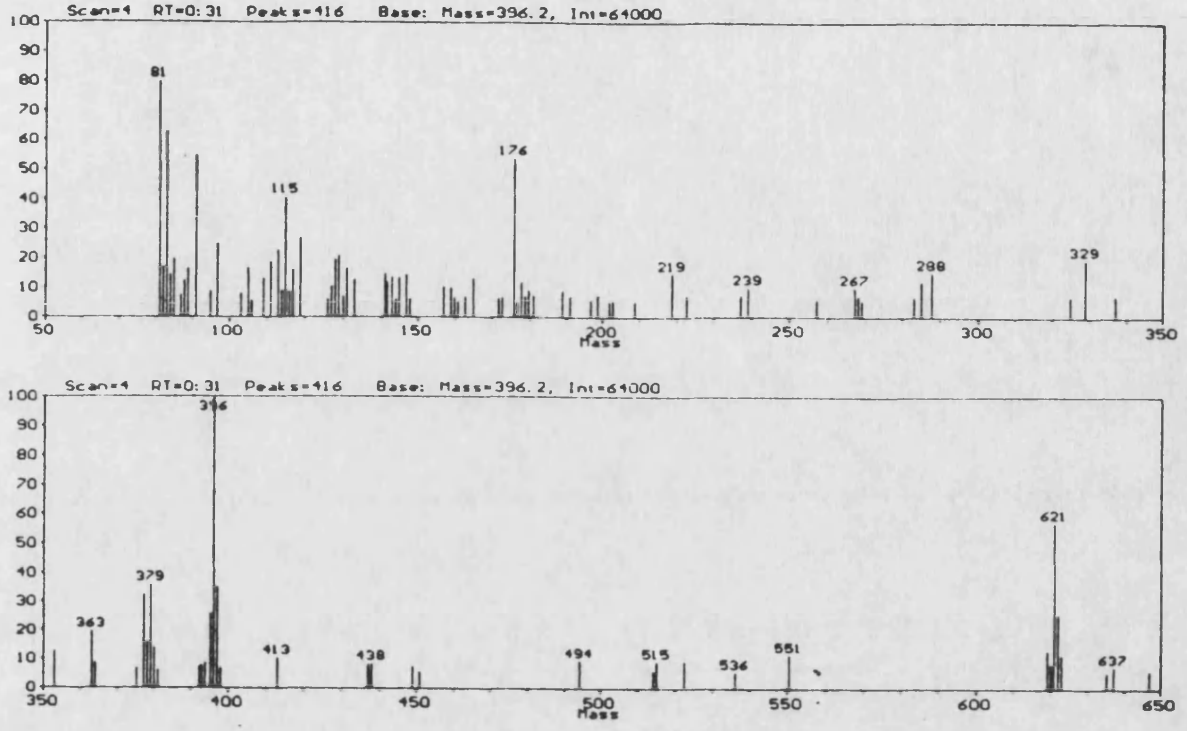
Figure 7

Positive ion fast atom bombardment mass spectra of standard and extracted ergosterol separated by HPLC

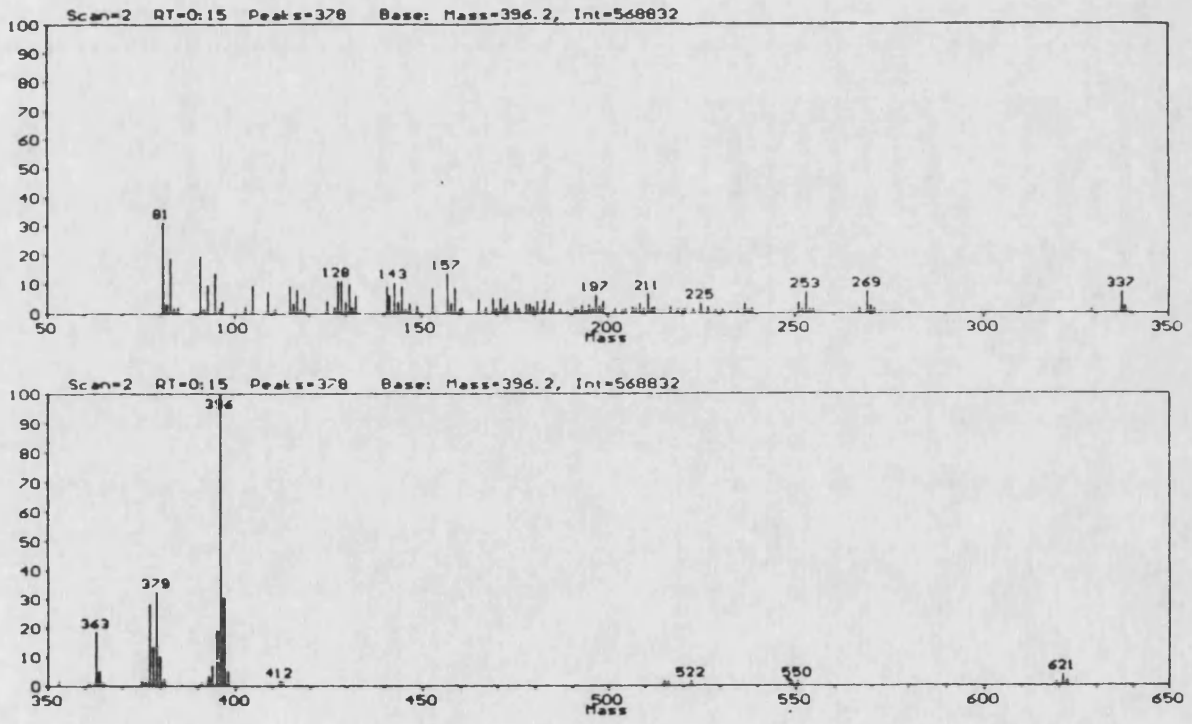
The ergosterol peaks were collected after separation as described in the text. The molecular ion for both standard (A) and extracted ergosterol (B) is 396.2. A contaminant at 621 is seen in both samples.

Figure 7

A


Scan=4 RT=0:31 Peaks=416 Base: Mass=396.2, Int=64000
81
115
176
219
239
267
288
329
Mass
Scan=4 RT=0:31 Peaks=416 Base: Mass=396.2, Int=64000
396
379
363
413
438
494
515
536
551
621
637
Mass

B


Scan=2 RT=0:15 Peaks=378 Base: Mass=396.2, Int=568832
81
128
143
157
197
211
225
253
269
337
Mass
Scan=2 RT=0:15 Peaks=378 Base: Mass=396.2, Int=568832
396
379
363
412
522
550
621
Mass

have an identical molecular weight of 395.2 Da (positive fast atom bombardment adds a proton to the sample), confirming that the sterol being detected is ergosterol.

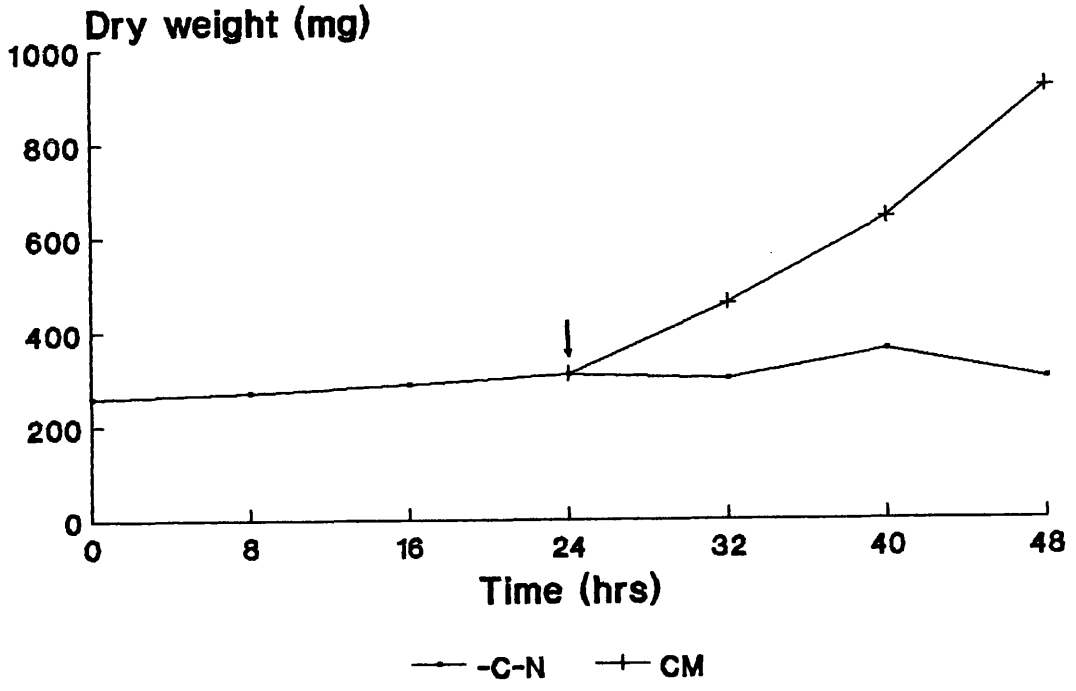
Sterols were extracted from 1g of insect cuticle and from elastin by the same method as for mycelium (section 2.2.3) to ensure no ergosterol was present in these inducing proteins; no peaks with retention times similar to ergosterol or ergocalciferol were detected.

Mycelium from 3-day CM cultures was transferred to -C-N basal salts medium and incubated for 24 hrs. The mycelium was then transfered to either CM (to give a level and rate of growth approximately equivalent to that on insect cuticle) or to -C-N basal salts (control) and incubated for a further 24 hrs. The dry weights (Fig. 8) and ergosterol content of the mycelia were measured at various times to confirm the presupposed relationship between ergosterol and fungal biomass (Fig. 9). The linearity of the graph shows that during the period of growth measured the ergosterol content remained constant at approximately 1.4 μg per mg dry weight of mycelium, thus enabling ergosterol to be used as a valid measure of fungal biomass in cultures containing insoluble substrates.

Extended periods of starvation for C and N resulted in a reduction in ergosterol yield per mg dry weight; after 36 and 48 hrs starvation ergosterol yields fell to 1.2 and 0.95 $\mu g\ mg^{-1}$

Figure 8

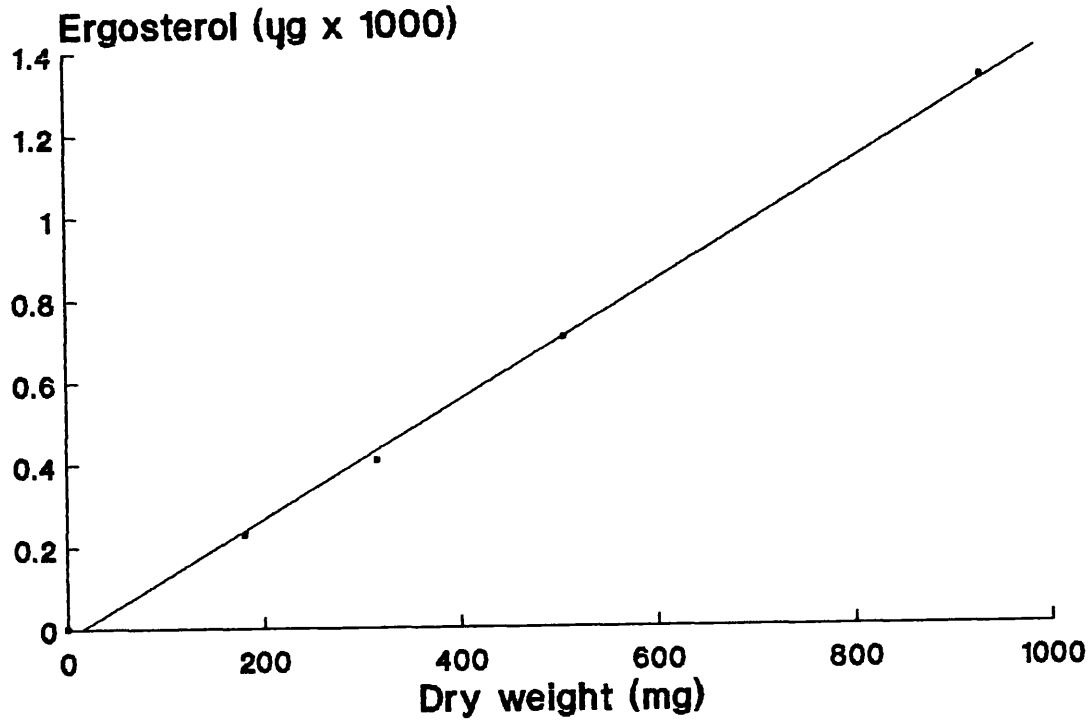
Mycelial dry weight of Metarhizium anisopliae in either complete medium or buffered basal salts (-C-N)



Mycelia from 3-day CM cultures (time zero) were starved of C and N for 24 hrs in buffered basal salts media before being transferred (arrowed) to CM or fresh buffered basal salts. Mycelial dry weights (means of three replicates) were determined after 8, 16, 24, 32, 40 and 48 hrs.

Figure 9

Relationship between fungal biomass (as dry weight) and ergosterol content



Ergosterol was extracted from cultures as described for Fig. 8 and quantified as described for Fig. 4.

respectively (see Table 6).

2) Evaluation of insect cuticle as an inducer of Pr1 production by comparison of fungal biomass.

Cultures containing cuticle, elastin or lacking C and N (control) were prepared as previously described. Elastin (which, similar to cuticle has a high alanine content) was also used in order to compare Pr1 production on an alternative insoluble protein substrate; also it is unlikely to cause C and N repression because of the insolubility of elastin. Ergosterol was extracted, and Pr1 activity measured 12 and 24 hrs after addition of either cuticle or elastin. The extracted ergosterol was quantified as described above and an estimation of fungal biomass was made (Table 6). Growth in control flasks was measured conventionally by dry weight determination, as extended periods of starvation result in reduced ergosterol yields.

After 12 hrs growth on cuticle, Pr1 activity was 10.4x higher than controls and 5.4x higher than elastin containing cultures. This corresponded with a 1.3- and a 1.1-fold higher estimate of biomass in cuticle cultures compared to control and elastin cultures respectively. After 24 hrs on cuticle, Pr1 activity was 11.3 and 10.5 times higher than controls and elastin cultures respectively, with biomass being 1.38- and 1.07- fold greater The high level of Pr1 activity on cuticle compared with controls occurred without a substantial increase in mycelial dry

Table 6

Fungal biomass and Pr1 production by *Metarhizium anisopliae*

Growth conditions	Ergosterol[1] (μg)	Dry wt. (mg)	Enzyme activity[2]	Activity mg^{-1}dry wt.
CM 3 Days	361	260	0	0
-C-N 24hrs	435	310	136.3	0.44
-C-N + 12hrs	396	330	201.4	0.61
-C-N + 24hrs	285	300	286.4	0.95
Cuticle[3] + 12hrs	600	429[4]	2090.6	4.87
Cuticle + 24hrs	975	696[4]	3245.2	4.66
Elastin[3] + 12hrs	540	386[4]	384	0.99
Elastin + 24hrs	855	611[4]	307.9	0.5

All results represent means of three replicates.

[1]Ergosterol extracted from mycelium and quantified as described in the text.

[2]Mean activity expressed as nmol NA $ml^{-1}min^{-1}$ liberated from Suc-$(Ala)_2$-Pro-Phe-NA.

[3]Potential inducers added to mycelium starved of C an N for 24 hrs.

[4]Dry weights estimated from ergosterol value.

weight and suggests that elevated Pr1 levels do not result from increased fungal biomass. Analysis of enzyme activity per mg dry weight shows that protease production in cuticle cultures increased ca. 8 and 5 fold after 12 and 24 hrs growth compared with controls and 5 and 9 fold compared with elastin grown cultures. Partial mycelial autolysis and subsequent release of intracellular enzymes may have caused the slight increase in Pr1 activity seen in control (-C-N) flasks because a slight fall in mycelial dry weight was observed: thus comparisons of Pr1 activity per mg dry weight are probably more valid after 12 hrs than 24 hrs growth.

Thus, the substantial increase in Pr1 production on cuticle is not a function of fungal growth because a similar increase in fungal biomass occurred with elastin grown cultures without a corresponding increase in Pr1 activity. These results cannot be explained in terms of derepression alone and therefore confirm that Pr1 is induced by insect cuticle.

3) Evaluation of protein as an inducer of Pr2 production by comparison of mycelial dry weight.

Earlier experiments have suggested that Pr2 may be induced non-specifically by proteins. To confirm the hypothesis that Pr2 production was not related to growth; enzyme production was facilitated by the use of BSA which gives high levels of Pr2 and allows the determination of mycelial dry weight.BSA was added to

cultures starved of C and N for 24 hrs as described previously, Pr2 activity and mycelial dry weights were measured after 12 and 24 hrs (Table 7).

After 12 and 24 hrs growth, Pr2 activity was 15.3 and 11.6 fold greater in cultures containing BSA than those in control (-C-N) cultures. This corresponds to an increase in activity per mg dry wt. of 14.7 and 5.6 respectively. After 48 hrs starvation (-C-N +24 hrs) mycelial dry weight had fallen but enzyme activity continued to increase; which may reflect some mycelial autolysis and a corresponding release of intracellular Pr2.

It is apparent that basal synthesis of Pr2 is controlled by derepression, but induced production (ca. 15-fold higher levels) occur in the presence of protein under derepressed conditions.

3.1.7. Cuticle components as inducers of Pr1 production.

Insect cuticle is composed of two layers, the outer epicuticle and the procuticle. The epicuticle is a composite structure which can be considered as a tightly cross-linked network of lipids and proteins (Anderson, 1979). The procuticle constitutes the majority of the cuticle and comprises chitin fibrils embedded in a protein matrix, together with lipids and quinones (Neville, 1984). Protein can contribute as much as 70% of the cuticle with chitin levels usually between 20 and 50%. It is therefore likely that Pr1 is induced by either the lipid, protein

Table 7

Fungal biomass and Pr2 activity in Metarhizium anisopliae

Growth conditions	Dry wt. (mg)	Enzyme activity[2]	Activity mg^{-1}dry wt.
CM 3 Days	290	0	0
-C-N 24hrs	305	21.2	0.07
-C-N + 12hrs	318	26.9	0.08
-C-N + 24hrs	283	45.4	0.16
BSA + 12hrs	398	411.1	1.03
BSA + 24hrs	593	526.8	0.89

All results represent means of three replicates.

[1]Mean activity expressed as nmoleNA $ml^{-1}min^{-1}$ liberated from Bz-Phe-Val-Arg-NA.

[2]BSA added to mycelium starved of C an N for 24 hrs.

or chitin component of cuticle.

To test this hypothesis cuticle, modified cuticle or cuticle components were added to cultures starved of C and N for 24 hrs; Pr1 activity was then measured after 4 and 12 hrs (Table 8). The treatments comprised, i) purified chitin (KOH washed as described in section 2.1); ii) deproteinated (KOH washed) cuticle and cuticle extracted with iii) ether or with iv) chloroform (to extract the lipid components, see section 2.1.2).

Ether and chloroform-extracted cuticle induced Pr1 production to a level similar to that with untreated cuticle, demonstrating that cuticular lipids do not affect Pr1 production. Chitin did not induce Pr1 but levels of activity nearly 2.5x that of controls (-C-N) were observed. It is possible that chitin was utilised as a souce of C and N enabling slightly enhanced Pr1 production without causing catabolite repression, in a way similar to that found with elastin (Fig. 1). Growth measurement by ergosterol estimation would have helped to clarify this possibility. Deproteinised cuticle also failed to induce Pr1, and resulted in levels of activity similar to those with chitin (possibly due to the chitin in the deproteinised cuticle). These results imply that the inducer of Pr1 is some component of cuticular protein. Deproteinised cuticle also failed to induce Pr2 (data not shown), further demonstrating the need for protein in Pr2 induction.

Table 8

Pr1 induction by components of insect cuticle by Metarhizium anisopliae.

	Enzyme activity[1]	
C/N source (1%)	4hrs	12hrs
Control (-C-N)	181.8 ± 6.9	290.9 ± 3.7
Cuticle	738.6 ± 21.5	2551.6 ± 58.9
Ether cuticle	545.5 ± 19.4	2863.7 ± 80.3
Chloroform cuticle	637.5 ± 17.4	2687.7 ± 61.7
Chitin	418 ± 26.1	711.4 ± 22.9
KOH cuticle	252.3 ± 11.5	593.2 ± 30.8

[1]Mean activity ± SD from 3 replicates 4 and 12 hrs after the addition of potential inducers to cultures starved of C and N for 24 hrs. Cuticle extracted with solvents as described in section 2.1.2 Enzyme activity expressed as nmolNA $ml^{-1}min^{-1}$ liberated from Suc-$(Ala)_2$-Pro-Phe-NA. The results are representative of three similar experiments.

3.1.8. Cuticular protein as an inducer of Pr1 production.

The component of insect cuticle which induces Pr1 production must be small enough to enter the cell. It is possible that the inducing peptide is hydrolysed from cuticle by a protease produced under conditions of derepression. From their studies with N. crassa Wolfinbarger and Marzluf (1975) have suggested that the oligopeptide transport system of filamentous fungi is unable to accommodate peptides exceeding the hydrodynamic volume of trileucine.

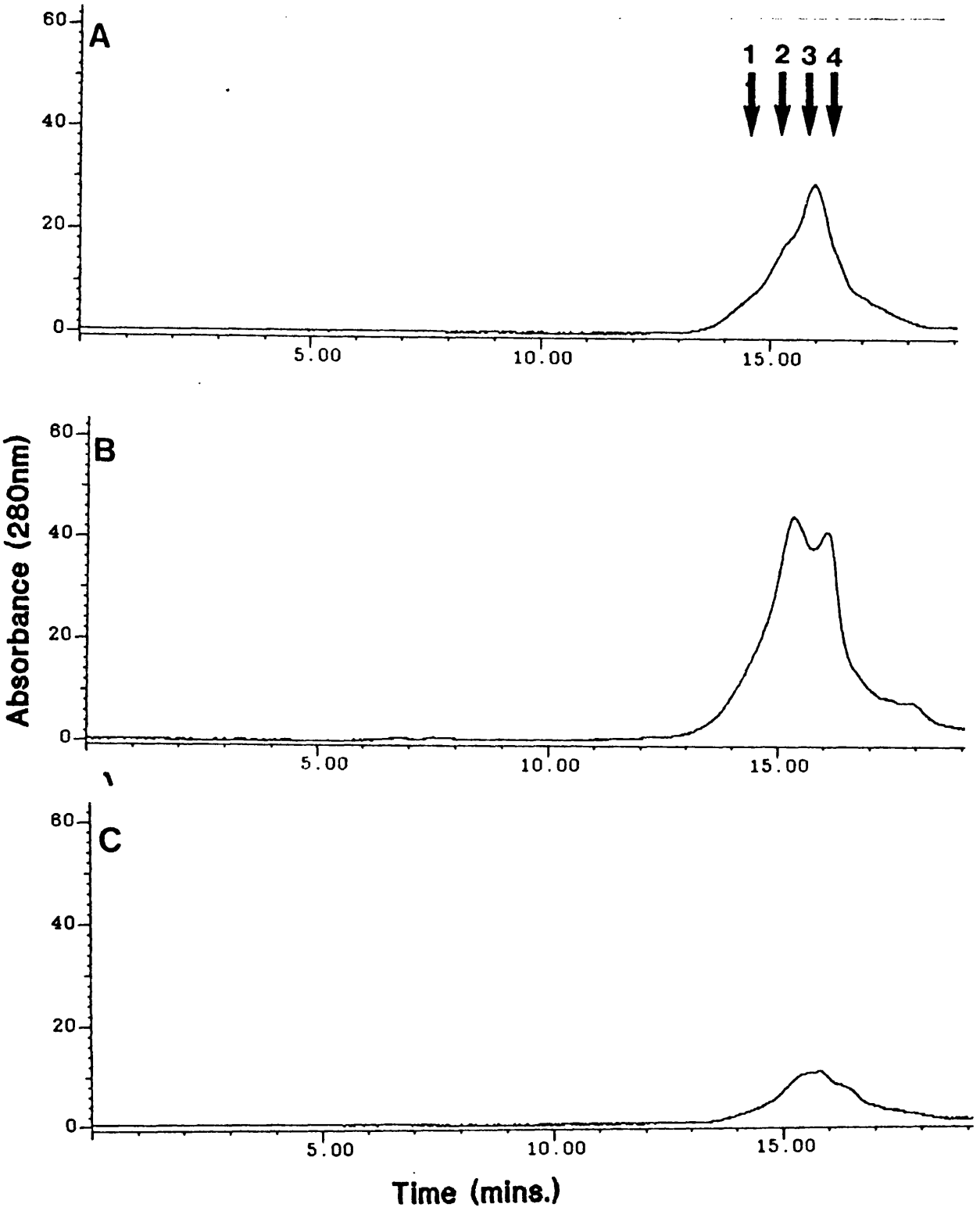
Attempts were made to obtain the peptides hydrolysed by the two proteases which could function in this regulatory capacity. One gram of cuticle was digested in 100 ml buffered basal salts (as used in induction experiments) containing 1 mg ml^{-1} of purified Pr1 or Pr2. The products were analysed by HPLC using an Anagel TSK 3000 SWXL gel filtration column (7.8 mm x 30 cm). The mobile phase was 0.1M Na_2SO_4 in 0.1M sodium phosphate buffer, pH 7.5 with a flow rate of 0.8 ml min^{-1}. The column was calibrated with L-phenylalanine, L-alanyl-L-proline, glutathione and insulin A (M_r 165, 186, 307 and 2,530 Da respectively). The two chromatograms with a buffer control are shown in Fig.10. Pr1 released peptides mainly in the range 150-2000 Da with a predominant peak at approximately 200 Da. Pr2 generated peptides of a similar size range to Pr1 but with a different distribution; peaks were at approximately 300 and 160 Da. Some peptides were solubilised in buffer alone, these were in the range 100-300 Da

Figure 10

High performance gel filtration of cuticle digests

Peptides released by the digestion of cuticle by Pr1 (A), Pr2 (B) or solubilised by buffer (C). Purified Pr1 or Pr2 (1 mg ml^{-1}) was added to 1g of cuticle in 100ml buffered basal salts medium and incubated at 27°C, 150 rpm for 24 hrs. The same conditions were used to solubilise peptides with buffer without the addition of enzyme. The peptide products were detected by absorbance at 280 nm. The system utilised an Anagel TSK 3000 SWXL column, with a mobile phase of 0.1 M sodium phosphate buffer, 0.1 M Na_2SO_4, pH 7.5 run at 0.8 ml min^{-1}. Calibration was as described in the text, with the molecular mass markers (numbered arrows) (1) 2531; (2) 307; (3) 186 and (4) 165 Da.

Figure 10



and were released in smaller quantities as shown by the reduced peak area. Both Pr1 and Pr2 hydrolyse peptides small enough to enter cells and function as potential inducers, but it is possible that the peptides solubilised by the buffer could also play a role in Pr1 induction in vitro.

To test the ability of the peptides to induce Pr1 the digestion products were first quantified by assaying for amino groups (ninhydrin assay, see section 2.3.9) and comparison with a standard curve constructed with L-alanine (see Appendix 1). The Pr1, Pr2 and buffer solubilised products contained 24, 17.5 and 9.5 μg alanine equivalents ml^{-1}respectively. Mycelium starved of C and N as described earlier was transferred to 100 ml basal salts medium containing 1.5, 3 and 4.5 μg equivalents ml^{-1} of either the Pr1, Pr2 or buffer solubilised peptides and Pr1 activity was assayed after 4 and 8 hrs (Table 9).

Addition of the peptides generated by Pr1 and Pr2 at 3 μg alanine equivalents ml^{-1} induced Pr1 production to a level similar to that seen with insect cuticle after 4 hrs growth. Also, after 12 hrs growth, the Pr2-generated peptides induced Pr1 to a level similar to that by insect cuticle, but the Pr1-peptide digest enhanced enzyme activity to a level ca. 66% of that seen with insect cuticle. Addition of buffer solubilised peptides at this concentration also enhanced Pr1 production, but to a lesser extent. Addition of all products at 4.5 μg alanine equivalents ml^{-1} resulted in a reduction in activities compared

Table 9

Effect of peptides generated from insect cuticle on Pr1 production by *Metarhizium anisopliae*

	Enzyme activity[1]	
	4hrs	8hrs
Controls		
-C-N	185.4 ± 16.9	268.3 ± 23.4
Cuticle 1%	485.7 ± 49.7	1297 ± 64.5
Pr1 products		
1.5 $\mu g\ ml^{-1}$	375.6 ± 25.4	607.4 ± 39.1
3 $\mu g\ ml^{-1}$	400.1 ± 19.2	818.7 ± 40.8
4.5 $\mu g\ ml^{-1}$	214.4 ± 11.5	482.2 ± 17.6
1:10	413.6 ± 24.4	791.7 ± 51.8
Pr2 products		
1.5 $\mu g\ ml^{-1}$	354.6 ± 17.4	849.9 ± 25.9
3 $\mu g\ ml^{-1}$	427.2 ± 33.1	977.2 ± 63.3
4.5 $\mu g\ ml^{-1}$	271.6 ± 22.6	662.3 ± 29.7
1:10	394.7 ± 26.6	1090.1 ± 71.8
Soluble products		
1.5 $\mu g\ ml^{-1}$	118.2 ± 16.4	374.3 ± 21.2
3 $\mu g\ ml^{-1}$	227.2 ± 17.5	413.6 ± 29.7
4.5 $\mu g\ ml^{-1}$	97.1 ± 6.8	428.1 ± 27.9
1:10	45.5 ± 9.5	113.7 ± 11.1

Results represent means of three replicates and are representative of three similar experiments.
[1]Enzyme activity expressed as nmolNA ml^{-1} min^{-1} liberated from Suc-$(Ala)_2$-Pro-Phe-Na.
[2]Mycelium starved of C and N for 24 hrs transferred to medium containing various concentrations of peptides generated from insect cuticle (as described in the text).

to those detected at 3 μg alanine equivalents ml^{-1}, probably as a result of catabolite repression.

To ensure that the slight enhancing effect of the buffer solublised products was not contributing to the induction effect seen with the Pr1 and Pr2 digests mycelia starved of C and N were transferred to flasks containing 10 mls (ie. a 1:10 dilution) of the digestion products released by either enzyme or by buffer. Pr1 induction was seen in flasks containing the Pr1 or Pr2 products but not the buffer solubilised peptides, suggesting that the induction seen in cuticle containing cultures is not due to the passive release of solubilised peptides, but occurs as a result of protease action on cuticle proteins. Pr1 and Pr2 release peptides with a mean chain length of 4.7 (St. Leger et al., 1986c) and 2.2 (St. Leger, 1986) respectively both of which are theoretically small enough to effect induction.

The profile of Pr2 generated products shows two peaks at approximately 160 and 300 Daltons. A sample of these peptides, equivalent to 87.5 μg alanine, was separated into two fractions, one approximately $\geq$ 200 Daltons and the other $\leq$ 200 Daltons and added separately to mycelium starved of C and N for 24 hrs. The fraction $\leq$ 200 Daltons ($\approx$ monomer size) completely repressed Pr1 production whilst the fraction containing peptides induced Pr1 to a similar level to that detected with insect cuticle. An amino acid analysis of this fraction is required to give some clue to the primary structure of the inducing peptide.

3.1.9. Potential peptide inducers from insect cuticle

Abundant amino acids and/or peptides that are unique to and common in host insect cuticle are possible candidates to effect Pr1 induction. Alanine is the predominant (ca 35%) amino acid in locust cuticle (St. Leger et al., 1986c) but little information is available on the primary structure of cuticular proteins. However, the N-terminal sequences of 8 proteins and the entire sequence from one protein from Locusta migratoria have been determined (Hojrup et al., 1986a, 1986b). The N-terminal sequence of all but one of the proteins is enriched with glycine with the remainder of the protein dominated by alanine which occurrs in characteristic motifs. Thus, the sequence data available indicates that Ala-Ala-Pro, Ala-Ala-Pro-Ala/Val and Ala-Ala-Ala are repeated regularly throughout large stretches of the proteins. It is possible that either alanine, glycine or one of the repeated peptides (complete or partial) could induce Pr1 production.

Mycelia starved of C and N for 24 hrs as described previously were supplied with the following potential inducers: alanine, glycine, Ala-Ala, Ala-Pro and Pro-Ala by means of diffusion capsules (see section 2.2.1) and at 0.1 and 0.01%. Ala-Ala-Ala was supplied at 0.1 and 0.01% only. The concentrations of amino acids and peptides were kept low in an attempt to avoid C and N repression.

Pr1 production under these conditions is summarised in Table 10. The concentration of amino acids detected in culture fluids from cultures containing diffusion capsules was 2.9 and 2.3 $\mu g\ ml^{-1}$ for alanine and its dimer respectively indicating that they were being utilised by the fungus at approximately the rate of diffusion and were not rising to levels likely to cause catabolite repression.

None of the amino acids or peptides induced Pr1 to a level equal to that on cuticle suggesting that the inducer is not one of the amino acids or peptides tested here. The monomeric amino acids increased Pr1 production to a greater extent than any of the peptides tested. This is probably due to a supply of readily utilised metabolites not sufficient to repress Pr1 production. The effects of catabolite repression were seen with all the dimers tested at 0.1%, but not with monomeric alanine or glycine; at first sight this is difficult to explain, but it is possible that Metarhizium has a more efficient uptake system for dimers than for the monomers. A comparison of initial growth rate on these peptides could confirm this. All the amino acids and peptides repressed Pr1 production when added at 1% (data not shown).

Table 10

Effect of potential inducers on Pr1 production by *Metarhizium anisopliae*

	Enzyme activity[1]	
C/N source[2]	4hrs	12hrs
Control (-C-N)	172.8 ± 11.4	440.6 ± 19.7
Cuticle	620.1 ± 37.6	2560.9 ± 69.7
Ala/restricted[3]	240.4 ± 28.6	726.7 ± 35.4
Ala 0.1%	221.5 ± 15.2	894.7 ± 41.6
Ala 0.01%	243.6 ± 33.7	897.3 ± 59.6
Gly/restricted[3]	309 ± 25.3	1082.8 ± 75.1
Gly 0.1%	314.4 ± 26.7	914.3 ± 41.8
Gly 0.01%	356.1 ± 31.9	1178.4 ± 86.2
Ala_2/ restricted[3]	218.2 ± 19.6	650.5 ± 28.5
Ala_2 0.1%	0	327.5 ± 22.1
Ala_2 0.01%	239.3 ± 24.8	761.4 ± 31.8
Ala_3 0.1%	0	456.9 ± 39.7
Ala_3 0.01%	169.9 ± 15.6	579.7 ± 23.7

Cont.

Table 10 cont.

Effect of potential inducers on Pr1 production by Metarhizium anisopliae.

	Enzyme activity[1]	
C/N source[2]	4hrs	12hrs
Ala-Pro 0.1%	0	295.6 ± 17.9
Ala-Pro 0.01%	354.5 ± 28.4	776.1 ± 44.4
Ala-Pro/restricted[3]	277.2 ± 29.1	711.3 ± 51.7
Pro-Ala 0.1%	0	314.6 ± 14.6
Pro-Ala 0.01%	272.7 ± 21.9	698.4 ± 36.9
Pro-Ala/restricted[3]	316.8 ± 32.2	652.6 ± 41.4

Results represent means of three replicates and are representative of three similar experiments.

[1]Enzyme activity expressed as nmolNA ml^{-1} min^{-1} liberated from Suc-$(Ala)_2$-Pro-Phe-Na.

[2]C/N source aded to mycelium starved of C and N for 24 hrs.

[3]Restricted supply from diffusion capsules as described in the text.

3.1.10. Other enzymes produced under derepression and induction conditions.

One of the aims of investigating protease regulation in M. anisopliae was to establish culture conditions in which hyphae contained a relatively high proportion of protease specific mRNA for cDNA cloning. Both Pr1 and Pr2 are produced rapidly by mycelium starved of C and N followed by induction with cuticle. Unfortunately, the other characterised cuticle-degrading enzymes ie. Pr4, L-alanyl aminopeptidase and the endo chitinase were not synthesised (at least not secreted) during C and N starvation or during the subsequent period of growth on cuticle. This means that any cDNA library optimised for protease cloning could not be used to identify cDNA clones of the other cuticle-degrading enzymes.

Fungal growth on insect cuticle or protein may result in a more complex mixture of mRNAs in the cell than in mycelia in media designed to produce protease by derepression alone which may complicate any cDNA cloning strategies. To investigate this possibility the enzyme profile of strain ME1 grown under derepression and induction conditions were examined.

Mycelia from 3-day CM cultures were starved of C and N for 24 hrs before the addition of elastin or cuticle (1% w/v). The effect of the starvation period was investigated by transferring mycelium straight from CM to basal salts plus cuticle without a period of

starvation. The enzyme activities of the culture filtrates were measured using the semi-quantitative API-ZYM system (Slots, 1981), which allows the rapid and systematic study of 19 enzymatic reactions (see section 2.3.2). The enzyme reactions and activities from filtrates 8 and 16 hrs after transfer are shown in Table 11.

Filtrates from all cultures exhibited high levels of alkaline and acid phosphatase and also phosphoamidase. Cultures starved of C and N for 24 hrs produced no other enzymes. Extended periods of starvation resulted in the production of N-acetyl glucosaminidase and small amounts of α and β glucosidase. Addition of elastin or cuticle to starved mycelia invariably resulted in more complex enzyme profiles; the main difference being the production of the proteases, trypsin and aminopeptidase. There was little difference between the enzyme profiles of cultures with added elastin or cuticle. Filtrates from cultures which had not been starved before the addition of cuticle had similar profiles to equivalent starved cultures but the levels of activity tended to be lower, presumably due to the effects of catabolite repression). Surprisingly, cuticle grown cultures produced notably less N-acetyl glucosaminidase than either starved or elastin grown cultures.

The extracellular protein profiles of some filtrates were also examined by PAGE. 100 μg of protein from filtrates of mycelia starved of C and N for 24 hrs, 8 and 16 hrs after the addition of

Table 11.

Reactions in the API ZYM test of culture filtrates of *M. anisopliae* under derepression or growth on cuticle and elastin

			-C-N		Elastin	
No.	Enzyme assayed	24hr	+8hr	+16hr	+8hr	+16hr
1	Control	0	0	0	0	0
2	Phosphatase alkaline	5	5	5	5	4
3	Esterase (C 4)	0	0	0	0	0
4	Esterase lipase (C 8)	0	0	0	0	0
5	Lipase (C 14)	0	0	0	0	0
6	Leucine aminopeptidase	0	0	0	0	3
7	Valine aminopeptidase	0	0	0	1	3
8	Cysteine aminopeptidase	0	0	0	0	1
9	Trypsin	0	0	1	1	2
10	Chymotrypsin	0	0	0	0	0
11	Phosphatase acid	5	5	5	5	5
12	Phosphoamidase	5	5	5	5	5
13	α galactosidase	0	2	3	0	1
14	β galactosidase	0	0	0	0	0
15	β glucuronidase	0	0	0	0	0
16	α glucosidase	0	0	1	1	1
17	β glucosidase	0	0	1	0	1
18	N-acetyl-β glucosaminidase	1	5	5	0	1
19	α mannosidase	0	0	0	0	0
20	α fucosidase	0	0	0	0	0

Table 11 cont.

Reactions in the API ZYM test of culture filtrates of *M. anisopliae* under derepression or growth on cuticle and elastin

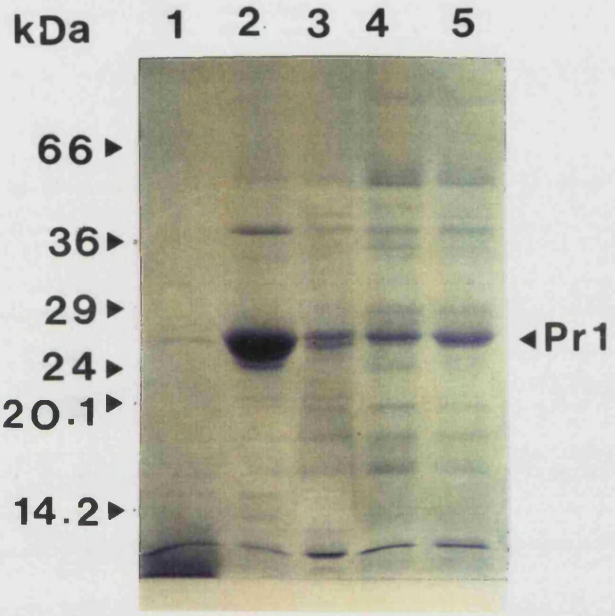
		Cuticle		CM - Cuticle[1]	
No.	Enzyme assayed	+8hr	+16hr	+8hr	+16hr
1	Control	0	0	0	0
2	Phosphatase alkaline	5	3	4	3
3	Esterase (C 4)	1	1	1	1
4	Esterase lipase (C 8)	0	0	0	0
5	Lipase (C 14)	0	0	0	0
6	Leucine aminopeptidase	4	4	1	3
7	Valine aminopeptidase	3	4	1	2
8	Cysteine aminopeptidase	2	2	1	1
9	Trypsin	1	3	1	2
10	Chymotrypsin	0	0	0	0
11	Phosphatase acid	5	5	5	5
12	Phosphoamidase	5	5	5	5
13	α galactosidase	0	1	0	0
14	β galactosidase	0	0	0	0
15	β glucuronidase	0	0	0	0
16	α glucosidase	0	1	0	0
17	β glucosidase	3	1	3	3
18	N-acetyl-β glucosaminidase	3	2	1	1
19	α mannosidase	0	0	0	0
20	α fucosidase	0	0	0	0

[1]Mycelium transferred directly from CM to cuticle without a starvation period.
Api Zym performed and quantified as described in section 2.3.2.

cuticle, 16 hrs after the addition of elastin and 16 hrs after transfer from CM to basal salts plus cuticle (no starvation) was loaded onto a 12% polyacrylamide gel (Fig. 11). The pattern of protein bands becomes more complex after the addition of elastin or cuticle, indicating that the fungus is synthesising many more extracellular proteins at this time which could complicate attempts to obtain cDNA clones of cuticle degrading proteases. However, the relative intensity of the Pr1 band from filtrates of cultures 16 hrs after the addition of cuticle to starved mycelia suggests that a large proportion of the mRNA in the cell at this time may be specific to Pr1.

Figure 11

SDS-PAGE of culture filtrates from derepressed and induced mycelia



100 µg of protein from filtrates of mycelia starved of C and N for 24 hrs (lane 1), 8 (lane 5) and 16 (lane 2) hrs after the addition of cuticle, 16 hrs after the addition of elastin (lane 3) and 16 hrs after the transfer from CM to basal salts plus cuticle (lane 4) analysed by electrophoresis on a 12% acrylamide gel. The gel was stained with Coomassie brilliant blue R250.

3.2. DISCUSSION.

The majority of enzymes with exogenous substrates are inducible (Jacob and Monod, 1961) and most inducible enzymes are subject to catabolite repression (Zubay et al., 1970). It has previously been reported that synthesis of Pr1 and Pr2 by M. anisopliae occurs rapidly by carbon and nitrogen derepression alone (St. Leger et al., 1988b). The work described here demonstrates that the regulation of these proteases is more complex and that an inducer - repressor mechanism, similar to that which controls chitinase production in M. anisopliae (St. Leger et al., 1986d) also operates. A number of authors have suggested that protease regulation in fungi can be controlled by both positive (induction) and negative (derepression) mechanisms (reviewed by Cohen, 1980). Protease production in bacteria has also been shown to be, in general, subject to catabolite repression and induction (Law, 1980).

Depletion of sulphur compounds specifically derepresses synthesis of extracellular acid proteases in Aspergillus niger (Tomanaga et al., 1964) and the alkaline protease of A. oryzae is produced upon starvation for carbon (Klapper, et al., 1973). In A. nidulans starvation for one or more of carbon, nitrogen or sulphur results in the synthesis and secretion of neutral and alkaline proteases (Cohen, 1973). In a further study Cohen (1981) surveyed protease regulation by 25 strains of Aspergillus and showed that derepression alone resulted in protease production in

all the 22 strains which produced proteases. Three strains produced acid protease and the remainder produced both neutral and alkaline proteases as in A. nidulans. A similar form of protease regulation has been reported for Schizophyllum commune (Sessoms and Lilly, 1986), but only under conditions of nitrogen starvation. The possibility that these proteases were further induced by protein, as with Pr2, was not reported.

Other fungi require both nutrient deprivation and the presence of of an inducing protein to effect protease production. N. crassa produces both neutral and alkaline proteases if starved of carbon, nitrogen or sulphur when grown on a protein substrate as sole nitrogen source, but in the case of carbon starved cells the addition of an activating protease is required for immediate synthesis of exocellular protease (Drucker, 1975; Cohen et al., 1975). Lasure (1980) demonstrated that protease production in Mucor miehei is similarly induced by protein if starved of nitrogen, but not if starved of carbon or sulphur in the presence of nitrogen. As with Pr2 the nature of the inducing protein in these cases seems relatively unimportant, because both soluble and insoluble proteins will induce protease production, although certain soluble, globular proteins (eg. egg white and gamma globulins) will not induce proteases in N. crassa (Drucker, 1975). Pr1 appears to be unique in that thus far, only one substrate, ie. insect cuticle, will induce enzyme production.

The regulation of proteolytic enzymes of some fungi pathogenic to

mammals has also been investigated. Protease regulation by the dermatophytic fungus Microsporum canis was studied by O'Sullivan and Mathison (1971) who claimed that protease was induced by protein. Mycelium grown on casein hydrolysate, then transferred to medium containing insoluble casein produced protease after 5 hrs. The possibility of catabolite repression by casein hydrolysate was ignored, an error apparent in many other studies of protease regulation, making interpretation of their results impossible. In Candida, a pathogen causing candidosis in immunocompromised humans and other mammals, an extracellular acid protease has been reported as a virulence factor (Macdonald and Odds, 1983; Kwon-Chung et al., 1985). Most of the pathogenic Candida species (C. albicans, C. tropicalis, C. parapsilosis, C. rugosa, C. lusitaniae and C. lipolytica) produce an acid protease when grown with BSA as sole nitrogen source, although other proteins and peptide mixtures (tryptone and peptone at 0.2%) also serve as protease inducers (Crandall and Edwards, 1987; Banerjee et al., 1991). Like Metarhizium, C. albicans (-N) and C. lipolytica (-N,C or S) produce basal levels of protease by derepression alone (Ogrydziak et al., 1977; Ross et al., 1990), with enzyme levels increasing rapidly upon the addition of protein to cultures. In the case of C. albicans, protease levels increased 20-50 fold only 4 hrs after addition of BSA (Ross et al., 1990). Induced protease production in Metarhizium increases to a lesser extent, Pr1 levels increased ca. 10 fold 16 hrs after addition of cuticle and Pr2 levels ca. 10 fold 8 hrs after addition of BSA or cuticle.

There have been few reported studies of protease regulation in entomopathogenic fungi. It has recently been reported that the entomogenous fungi V.lecanii, B.bassiania, Tolypocladium niveum and Paecilomyces farinosus also produce a Pr1-like enzyme during nutrient deprivation (St. Leger et al., 1991b). The mosquito parasitising fungus Lagenidium giganteum produces both trypsin and collagenase proteases in the presence of an inducing protein substrate which are repressed by glucose (Dean and Domnas, 1983). B.bassiania, which produces proteases with both chymoelastase and trypsin like properties (St. Leger et al., 1987b) produces only the Pr1 like enzyme when grown with gelatin as sole carbon and nitrogen source (Bidochka and Khachatourians, 1988a). This contrasts with the findings in this study for Metarhizium which produces only Pr2 when grown in similar media. Kucera (1981) studied the proteolytic activity of M. anisopliae on a range of nitrogen sources and reported that after 12 days growth the highest activity was attained with Galleria mellonella proteins, but unfortunately did not take into account the effects of catabolite repression.

Pr2 is detectable 3-4 hrs after transfer of mycelium from CM to buffered basal salts medium whereas Pr1 appears after 6-8 hrs. Neither protease was detectable earlier if the basal salts medium contained cuticle. This might suggest that there are sufficient reserves of intracellular carbon and nitrogen to repress production of extracellular proteases for several hours. Introducing a period of starvation for a particular nutrilite

prior to the transfer of mycelium to cuticle cultures can affect the nature of protease production. Starving mycelium of carbon alone for ≥ 2 hrs resulted in little Pr1 activity even 12 hrs after transfer to cuticle medium, whereas Pr2 appears to be relatively unaffected by the period of carbon starvation. Starvation of nitrogen appears to be a major controlling factor for Pr1, as activity was proportional to the time of starvation for both carbon and nitrogen. Therefore, the difference in protease production when the fungus is starved of carbon alone or carbon and nitrogen appears to be a qualitative response and not due to a lack of availabilty of carbon within the cell. Presumably, germinating conidia could derive limited carbon from lipids on the surface of insect cuticle and still be in a state of derepresion; this would result in basal synthesis which should initiate higher induced synthesis of both Pr1 and Pr2. Starvation for carbon alone may also result in the production of other extracellular depolymerases such as carbohydrate degrading enzymes.

It is impossible to interpret the increase in enzyme activity found in cuticle containing cultures without an assessment of fungal growth in order to determine whether the rise in protease activity was due to induction or an increase in fungal biomass. Mycelium cannot be separated from insoluble substrates such as cuticle and elastin, which necessitated the use of an alternative to dry weight measurement for estimating fungal biomass.

Chitin, a cell wall component of most fungi is the marker most commonly used in cases of fungal growth on solid substrates (eg. Ride and Drysdale, 1972; Frankland et al., 1978). Chitin could be used to estimate fungal biomass in cultures containing pure protein (ie. elastin) but not insect cuticle, as cuticle contains between 20 and 40% chitin (Neville, 1975). An alternative marker for fungal biomass is Provitamin D_2 or ergosterol (24β-methylcholesta-5,7,trans 22-trien-3β-ol) which is the prominent sterol of most fungi (Weete, 1974); although there are exceptions such as Fujino and Ohnishis' (1979) report that A. oryzae contains brassicasterol (24β-methylcholesta-5,trans 22-dien-3β-ol) and not ergosterol. Seitz et al.(1979) obtained good correlation between chitin and ergosterol levels in A. flavus, A. amstelodami and Alternaria alternata. Ergosterol levels were reported to fluctuate less than chitin levels in Agaricus bisporus and therefore was a more reliable indicator of fungal biomass (Matcham et al., 1985). Consequently, ergosterol is gaining favour as a measure of fungal biomass (Seitz et al., 1977; Newell et al., 1987; Martin et al., 1990).

Sterols were extracted from M. anisopliae and ergosterol was identified by retention time (by reverse phase HPLC), spiking samples with pure ergosterol and comparison of mass spectra. No ergosterol was detected in extracts of cuticle or elastin. The ergosterol yield from mycelium grown in CM was 1.4 $\mu g\ mg^{-1}$ dry weight which compares favourably with other reported yields from fungi grown in similar conditions, eg. 2.3, 3.8 and 5.9 $\mu g\ mg^{-1}$

for A. alternata, A. flavus and A. amstelodami respectively (Seitz et al., 1979), 5 $\mu g\ mg^{-1}$ for A. nidulans (Shapiro and Gealt, 1982) and 1.9 - 2.5 $\mu g\ mg^{-1}$ in ascomycete salt marsh fungi (Newell et al., 1987). Extended starvation periods resulted in lower ergosterol yields from M. anisopliae of 0.95 - 1.2 $\mu g\ mg^{-1}$. A similar reduction in ergosterol yield (1.1 compared to 1.9 - 2.5 $\mu g\ mg^{-1}$) was found in ascomycete salt marsh fungi as a result of partial autolysis (Newell et al., 1987). The reduction in dry weight of mycelium starved of carbon and nitrogen for extended periods suggests that the low yields found in Metarhizium could also be a result of mycelial autolysis.

The total ergosterol extraction procedure described is time consuming and therefore not suitable for large sample numbers. However, consistent with the findings of Newell et al. (1988) it was found that samples could be stored for at least one month in methanol before analysis without any photoconversion of ergosterol to ergocalciferol (Tuksida, 1980). Martin et al. (1990) have recently described an improved assay which measures only free ergosterol (≈80%) following extraction with ethanol but not ergosterol esters (≈20%) which are released by saponification; this technique reduces the extraction and analysis time to a few minutes, and could be suitable for further studies on protease regulation in M. anisopliae. Measurement of DNA content could also be used to estimate fungal biomass (Giles and Myers, 1965) but obviously could not be used to estimate fungal invasion of whole insects, which is another potentially

useful application of the ergosterol assay, providing no ergosterol is extracted from host insects.

The ca. nine fold increase in Pr1 activity per mg dry weight in cuticle containing cultures compared to controls cannot be explained by derepression alone. Possible explanations are that only insect cuticle gives an optimum supply of carbon and nitrogen for protease production but insufficient to cause catabolite repression, or that a component of insect cuticle specifically induces Pr1. The fact that low levels, or slow feeding of amino acids or peptides via diffusion capsules and adding buffer solubilised peptides at a range of concentrations failed to cause a similar rise in Pr1 activity would suggest the latter. Also, in cultures with a range of carbon and nitrogen sources Pr1 activity did not approach the levels seen on cuticle would seem to reflect a specific effect. One "criterion of inducibility" is the detection of greater enzyme activity when the fungus is grown on insect cuticle than when grown on other non-repressing substrates; however the distinction between induced and constitutive synthesis can be marginal as the degree of inducibility (ie. ratio of induced : basal synthesis) can vary from ca. 1000 for β-galactosidase of E. coli to less than 10 for penicillin β-lactamases of Gram-negative bacteria (Smith, 1963).

The component of insect cuticle which induces Pr1 appears to be cuticular protein (or peptides thereof) as shown by (i) reduced Pr1 levels in cultures grown on deproteinised cuticle, (ii) other

cuticle components (ie. chitin and lipids) had no affect on induction and (iii) the induction of Pr1 by peptides released from cuticle by pure preparations of Pr1 and Pr2. These findings are in marked contrast with those of St. Leger et al. (1988b) who suggest that because of the enhancing effect of certain polymers, such as cellulose (an effect not apparent in this study), induction by cuticle proteins is probably not a major factor, but protease activity was only assayed for seven hours after transfer and therefore the effects of induction could have been missed.

The increase in Pr2 activity per mg dry weight when the fungus is grown on BSA as sole carbon and nitrogen source confirms that Pr2 is induced by BSA and probably by other proteins, but an assessment of growth on other proteins is required to confirm this possiblity.

These results show that Pr1 and Pr2 are regulated in a similar way, but the induction of Pr1 has evolved to occur only in the presence of insect cuticle, thus enabling the fungus to react rapidly and qualitatively to contact with the host insect. The complete repression of Pr1 by soluble proteins shows that Pr1 production is more sensitive to catabolite repression than Pr2 production, findings similar to those of St. Leger et al. (1988b).

In a less detailed study of enzyme production by five strains of M. anisopliae from diverse sources, Gupta et al. (1991) reported

that all five strains produced Pr2, but little Pr1 on media containing gelatin as sole carbon and nirogen source and that three of the five strains produced exceptionally high levels of Pr1 in cuticle grown cultures, with the other two strains producing no Pr1 at all. These authors suggested, but did not attempt to prove, that the differential production of proteases on cuticle and gelatin may be a result of substrate induction.The high levels of Pr1 occurred in cultures containing ground cuticle from the Greater Wax Moth (Galleria mellonella) or the Cabbage Looper (Trichpulsia ni), which suggests that the induction of proteases in ME1 demonstrated in this study is not a strain or host specific phenomenon as cuticle of lepidopteran origin can induce chymoelastase in strains isolated from coleopteran hosts.

To the authors knowledge, this is the first reported example of the specific induction of a protease in any microorganism. However, Geremia et al. (1991) reported that the mycopathogenic biocontrol agent Trichoderma harzianum produces a protease similar to Pr1 (high pI [9.2], with the same preferred peptide substrate but with no elastase activity) when grown on autoclaved mycelia of Botrytis cinerea and T. viride but not Rhizoctonia solani or Fusarium oxysporum. The authors concluded that the protease was specifically induced by fungi with a high protein content in their cell walls. This was not proven, however and it remains likely that the protease of T. harzianum may be induced by any protein (a possibility not investigated) as is the case with most induced proteases of fungi.

Of the few fungi studied, protease regulation by derepression and induction has only been reported for pathogenic species. Unfortunately, studies on Aspergillus species (Cohen, 1973, 1981) fail to report on whether protease levels rise when grown with protein as sole nitrogen source. It is therefore difficult to say whether this form of regulation is a pathogenic adaptation as protein may also have an inductive effect in Aspergillus. In a review of protein utilisation by fungi, Cohen (1980) questions whether the inductive effect of protein above basal levels of enzyme produced by derepression (as seen in this study and in C. albicans [Ross et al., 1990]) can indeed be termed induction, as protein is not strictly required for protease production. Enzyme regulation by derepressed basal synthesis and specific induction has also been reported for some cell wall degrading enzymes of the plant pathogens. Induction in these cases has been proven with the identification of specific monomeric and dimeric inducers. For the vascular wilt fungi Verticillium albo-atrum and Fusarium oxysporum synthesis of each cell wall degrading enzyme was induced rapidly by the monomer predominant in the substrate. Thus, polygalacturonan hydrolase and lyase were induced by galacturonic acid, arabinase by arabinose and xylanase by xylose (Cooper and Wood, 1973, 1975); but probably due to the abundance of glucose in nature, the inducer of cellulases in these and other fungi has been shown to be cellobiose and not the monomer (Mandels and Reese, 1960). A similar situation is found with amylase and the dimer maltose. Metarhizium produces high basal levels of Pr1 (ca. 10% of induced levels), this less relatively

loose control over synthesis together with the evidence that inhibition of Pr1 delays cuticle penetration (St. Leger et al., 1988a) might suggest a key role in pathogenesis. The soft-rot, plant pathogenic bacterium Erwinia chrysanthemi produces very high basal levels of inducible pectate lyase which alone is sufficient for pathogenesis, because a mutant non-inducible for the enzyme retains pathogenicity (Collmer et al., 1982).

Catabolic enzymes which attack exogenous substrates (eg. cell-wall degrading enzymes of plant pathogens) are, as a rule, induced by specific compounds structurally related to the enzyme's substrate or reaction products (Cooper, 1977, 1983). In the case of induced proteases the inducing substrate is a macromolecule which, in the absence of protein permeases (Cohen, 1980) should not be capable of entering the cell (Rogers, 1961). Wolfinbarger and Marzluf (1975a) showed that amino acid auxotrophs of N.crassa were unable to grow on peptides larger than pentapeptides and suggested that fungal cells (Neurospora specifically) might be restricted in growth on larger peptides by the inability of an oligopeptide transport system to accommodate peptides with a hydrodynamic volume larger than trileucine. These peptides were shown to be transported intact via the oligopeptide transport sytem as opposed to hydrolysis by extracellular proteases and transport of the resulting free amino acids (Wolfinbarger and Marzluf, 1975b). A similar transport system has been found in yeasts where the size exclusion limit appears to be variable between strains (Marder et al., 1977). If, as seems

likely, a similar transport system operates in Metarhizium then the inducing molecule is likely to be at or below the level of a pentapeptide.

Despite the fact that proteases of certain fungi, eg. Candida are induced by peptide mixtures, such as peptone and tryptone (Banerjee, et al., 1991) it is remarkable that the exact nature of the inducing molecule has not been identified for any microbial protease. Drucker (1972) reported an unidentified low molecular weight 'factor' in filtrates from cells of N.crassa induced with BSA which when added to repressed cultures caused a five fold increase in protease production. This 'factor' was heat stable and apparently not a simple product of BSA, but was unfortunately not mentioned in any subsequent publication.

Inducible proteases of fungi eg. Candida and N.crassa, like Pr2 are induced by a wide range of unrelated proteins. This could make the identification of an inducing peptide difficult unless it is the case that any peptide of a certain size will induce protease production; or analagous to polysaccharidases, monomers may be inducers if kept at non-repressing levels, but for proteases presumably many amino acids would be involved because of the heterogeneity of monomer composition of proteins (Cooper, 1977). Amino acids did not induce protease in Verticillium albo-atrum (Mussel and Strouse, 1971) or in M. anisopliae in this study.

A mechanism for the release of inducer(s) from cuticle proteins must be envisaged. Inducible enzymes are usually secreted to a small extent by cells in the absence of inducer (Cooper, 1977) and serve to release smaller, diffusible compounds (ie. the inducer) from the substrate. Such basal synthesis was not found for N.crassa (Drucker, 1975; Cohen et al., 1975). It has been suggested (Drucker, 1975; Cohen, 1980) that a mechanism of 'local induction' could operate whereby recognition of substrate protein would occur only when it is in contact with the cell, but presumably effector molecules would still have to be released. The amount of enzyme produced in basal synthesis can vary and it is possible that the general protease assay used in the studies with N. crassa was not sufficiently sensitive to detect a low level of basal synthesis.

The specificity of Pr1 induction by insect cuticle could make it easier to determine the nature of the inducing molecule than in those cases where induction is effected by a number of different proteins. The products of cuticle digestion by pure preparations of either Pr1 or Pr2 are capable of inducing Pr1 production when added to derepressed mycelium, but compounds solubilised by buffer do not, which demonstrates that a peptide(s) of cuticular protein is the inducing molecule(s). Induction appears to be an autocatalytic process in which the action of the two proteases produced at a basal level, participate in converting proteinaceous cuticle polymers to oligopeptide inducers.

Prima facie peptides unique to and common in host insect cuticle are likely candidates as potential Pr1 inducers from insect cuticle. The origin of host cuticle appears to be unimportant as cuticle from a number of species induces chymoelastase synthesis in M. anisopliae. Thus, any similarities between primary structures of cuticle proteins of different species of insects may give a clue to the amino acid sequence of the inducer(s). There is little published sequence information for exocuticle proteins, but the N-terminal sequences of 8 proteins and the entire sequences of 2 proteins from the exocuticle of L. migratoria have been elucidated (Hojrup, et al., 1986a, 1986b; Klarskov et al.,1989). The extreme N-terminal regions of all but 1 protein are glycine rich and large stretches of the remainder of the cuticle contain repetitive sequences dominated by alanine. Thus, in 'protein 38' from L. migratoria Ala-Ala-Pro is found 14 times, Ala-Ala-Ala 6 times, Ala-Ala-Pro-Ala 10 times and Ala-Ala-Pro-Val 6 times (Hojrup et al., 1986b); these repeated peptides constitute ca. 70% of the entire sequence. In the alanine rich regions, the proline residues are most frequently spaced with a distance of 6 residues. In the only other Locusta exocuticle protein sequenced the N-terminus is dominated by Ala-Ala-Pro-Ala/Val and the C-terminus by alanine and valine (Klarskov, et al., 1989). The primary structures of two low molecular weight proteins (probably of endocuticular origin) from L. migratoria has recently been reported (Nohr et al., 1992). One protein (NCP-55) contains 33 residues, in which the sequence Ala-Ala-Pro-Ala occurs twice. However, this sequence does not occur in the second

protein (NCP-62), although the sequence Gly-Val-Tyr occurs four times. Locusta and Schistocerca proteins appear to have very similar amino acid compositions (Andersen, 1988a). It is not known whether the repetitive sequences found in locust cuticle proteins are unique to Locusta or are also found in other species. One protein from the endocuticle of L. migratoria has also been sequenced which shows very little homology to the sequences of exocuticle proteins sequenced (Talbo et al., 1991), but it shows pronounced similarity to cuticular proteins from larvae of Diptera and Lepidoptera (Rebers and Riddiford, 1988). The most striking similarity is seen at the C-terminus where the sequence PTPPPIPPAI corresponds to PTPPPTVAI in Drosophila melanogaster (Snyder et al., 1982) and PTPPPIPAAI in Manduca sexta (Rebers and Riddiford, 1988). This proline rich region which is conserved over three orders of insect indicates a similarity between soft larval cuticle and locust endocuticle which may extend to mechanical properties (Talbo et al., 1991). Other structural proteins of insect (but not cuticular) origin such as the chorion proteins from silkmoth eggs (Hamodrakas et al., 1982) and the cockroach oothecal proteins (Pau, 1984) show little homology to cuticle proteins, but appear to be rich in glycine in terminal regions and have central regions rich in valine and alanine. Part of the sequence of the oothecins contains a repeated pentapeptide Gly-Gly-Leu-Gly-Tyr which is identical to tandemly repeated pentapeptides found in tyrosine-rich regions in silkmoth chorion proteins (Weldon and Kafatos, 1980).

It is possible that the inducer(s) of Pr1 released from cuticle is in some way related to the preferred cleavage sites of either Pr1 or Pr2. The best substrate of Pr1 is Suc-$(Ala)_3$-Phe-NA although the substrates Suc-$(Ala)_2$-Pro-Ala-NA, Suc-$(Ala)_2$-Pro-Phe-NA and Ac-$(Ala)_3$-NA are also readily hydrolysed (St. Leger et al., 1987a). These substrates bear a striking resemblance to the repetitive peptides seen in locust cuticle, which would be excellent substrates for Pr1, containing many cleavage sites. The action of basal levels of Pr1 on cuticle would therefore release many alanine rich peptides which could hypothetically induce further Pr1 production. Unlike many fungal proteases which have a broad specificity (North, 1982), Pr2 has a primary specificity for arginine and lysine comparable to that of bovine trypsin (St. Leger et al., 1987a). There are no obvious cleavage sites for Pr2 in the repetitive sequences in locust cuticle which contains only ca. 5% lysine and arginine (St. Leger et al., 1986c). Although presumably cuticle degradation by Pr2 would also release alanine rich peptides because alanine is the predominant amino acid in locust cuticle (St. Leger et al., 1986c).

Neither Ala, Gly, Ala_2, Ala_3 nor Ala-Pro dimers induced Pr1 to a level equivalent to that produced on cuticle, although elevated levels of activity were present with monomers; but in the absence of a determination of biomass a growth effect cannot be ruled out. St. Leger et al. (1986a) reported that both alanine and glycine were able to support growth of M. anisopliae, although alanine was utilised to a much greater extent. Wolfinbarger and

Marzluf (1974) showed that N. crassa did not grow well on dipeptides due the absence or low activity of a dipeptide transport system. Should Metarhizium also be inefficient in utilising dipeptides this could explain the low Pr1 production seen in dipeptide containing cultures and could preclude their investigation as possible inducers. An analysis of growth on peptides and monomers would confirm this.

Derepressed mycelium of Metarhizium produces mainly proteases when grown on protein or cuticle as shown by API ZYM. Analysis of culture fitrates by PAGE reveals that when cuticle is added to derepressed mycelium Pr1 is by far the most abundant extracellular protein. St. Leger et al. (1988b, 1991b) showed by addition of metabolic inhibitors to cultures and by in vitro translation that protease production is regulated at the level of transcription; ie. derepression results in de novo synthesis of mRNA. It would appear that inducing proteases with insect cuticle as described here could produce mycelium which contains abundant protease specific mRNA for use in cDNA cloning strategies, but a similar study, using inhibitors of transcription, translation and secretion needs to be carried for induced Pr1 in order to determine whether Pr1 induction results in de novo mRNA synthesis or an increase in the rate of translation and/or secretion.

4. CLONING OF PROTEASE GENES

4.1. RESULTS

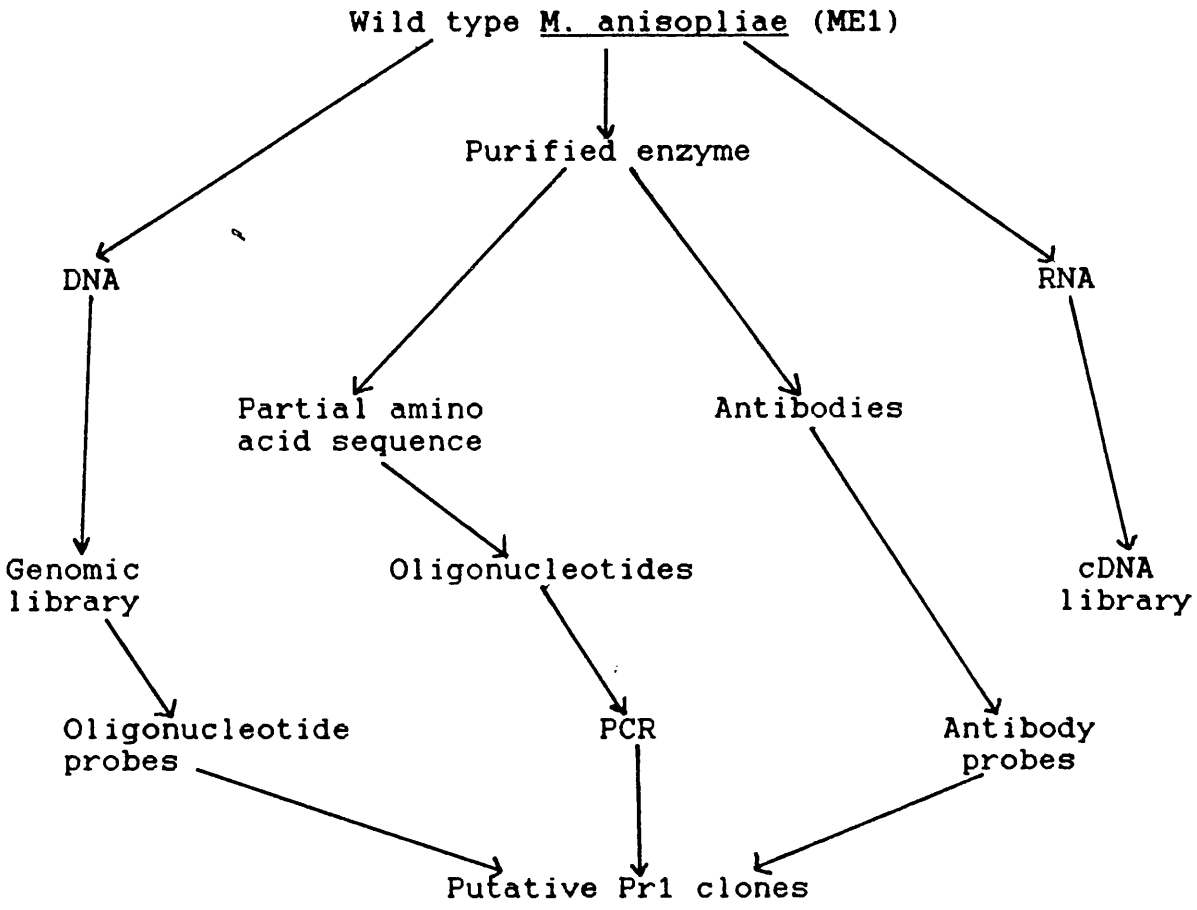
Previous attempts in this laboratory to produce protease deficient mutants of M. anisopliae by a variety of methods have proved unsuccessful (J.M. Clarkson, pers. comm.). This together with the low transformation frequency (0.1 transformants per μg DNA) attainable using current transformation systems (Bernier et al., 1989; Goettel et al., 1990) precluded the cloning of protease genes by complementation of mutants with random fragments of genomic DNA.

An alternative approach was to isolate clones of the genes encoding Pr1 and Pr2 from a genomic library by screening with oligonucleotides derived from protein sequence data obtained from purifying and sequencing the two enzymes. Antibodies vs Pr1 were also raised in an attempt to isolate cDNA clones from an expression library. A flow diagram demonstrating the attempted cloning strategies utilised for Pr1 is given in Fig. 12

4.1.1. Protease purification.

Proteases were purified from filtrates of 5-day cultures grown on 1% ground cuticle in basal salts medium. Mycelium and cuticle were removed by filtration over a sintered glass funnel and the filtrate concentrated ca. ten-fold by ultrafiltration (YM5

Fig. 12 Cloning strategies for Pr1.



Flow diagram showing the methodology used in the cloning of the Pr1 gene.

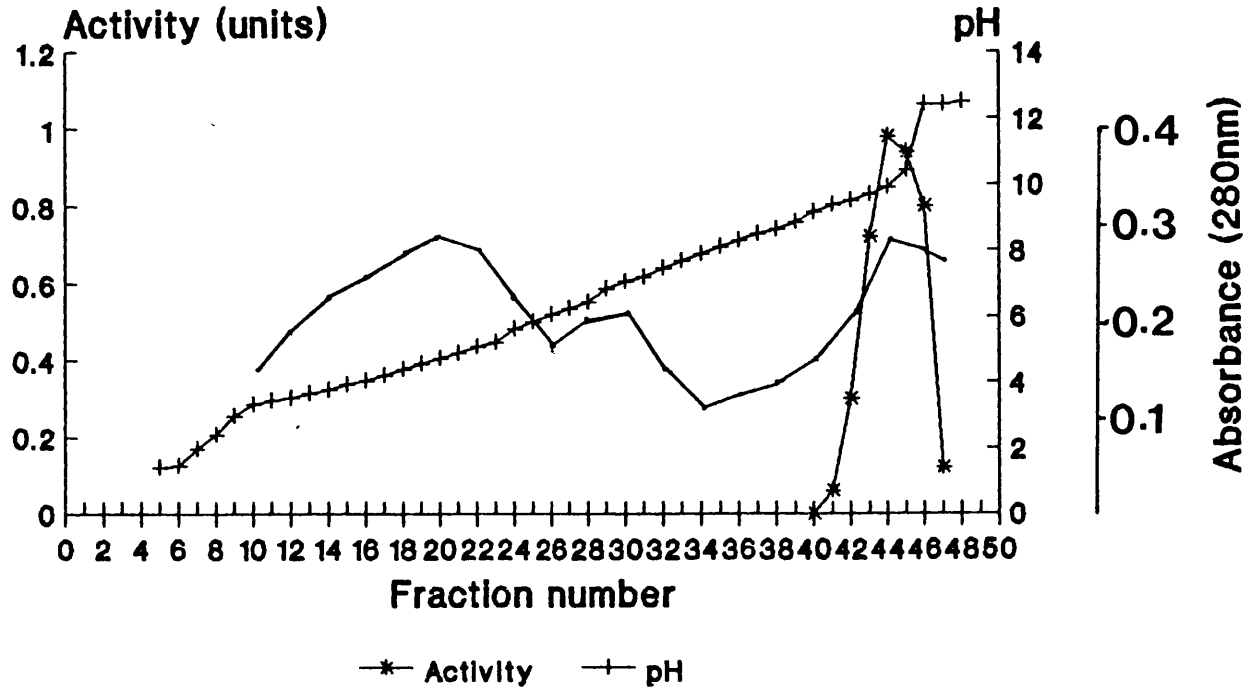
filter, Amicon) at 4°C. The concentrated extract was then used to purify Pr1 by either IEF or HPLC and Pr2 by anion exchange chromatography.

Pr1 was initially purified essentially by method described by St. Leger et al. (1987a). The concentrated filtrate was subjected to wide range IEF (pH 3-10) to separate Pr1 (pI > 10) from other proteins (Fig. 13). Ampholytes and small contaminating proteins (later found to result from autodigestion) were subsequently removed from fractions (43-46) containing Pr1 by Sephadex G-100 chromatography (Fig. 14). Pr1 was later purified by cation exchange HPLC (devised in collaboration with Dr. S. Cole) as this method resulted in a purer preparation and was less time consuming. Separation was performed on a Dynamex SCX-Ti (Rainin) strong cation exchange column (4.6 mm x 10 cm) equilibrated with 20 mM Na acetate (1 ml min^{-1}), pH 5.5. Elution of basic proteins was achieved with a linear gradient (0-0.4 M NaCl) in similar buffer with detection at 280 nm. The majority of the proteins in the concentrated filtrate did not bind to the column; with Pr1 and its pro-enzyme usually being the only proteins eluted by the gradient (Fig. 15). Fractions containing Pr1 were immediately placed on ice to prevent autodigestion by the protease.

Pr2 has been previously purified by wide range (pH 3-10) and then narrow range IEF (pH 2.5-5) St. Leger et al. (1987a). In this study, Pr2 was purified by anion exchange chromatography on Q-Sepharose; this gives is a more rapid separation and the

Figure 13

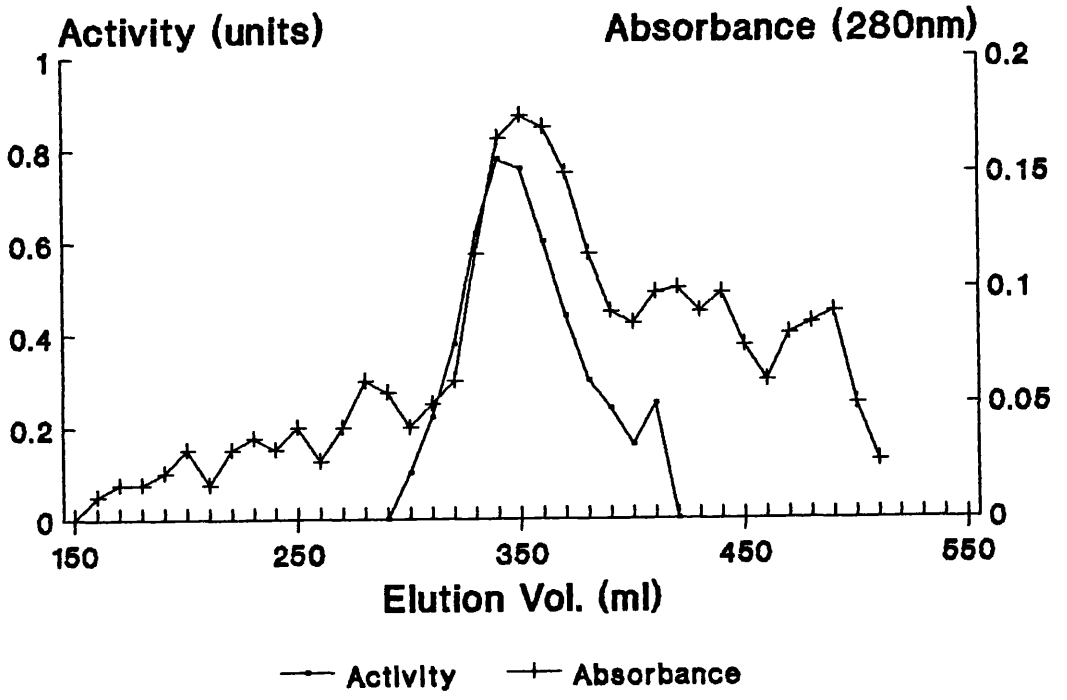
Purification of Pr1 by preparative isoelectric focusing



The experimental conditions are described in the text. Protein was estimated by absorption at 280 nm (—•—). The pH was determined with a glass combination electrode. One unit of activity liberates 1 μmole NA $min^{-1}\mu l^{-1}$ from Suc-$(Ala)_2$-Pro-Phe-NA. Fraction size was 9 ml.

Figure 14

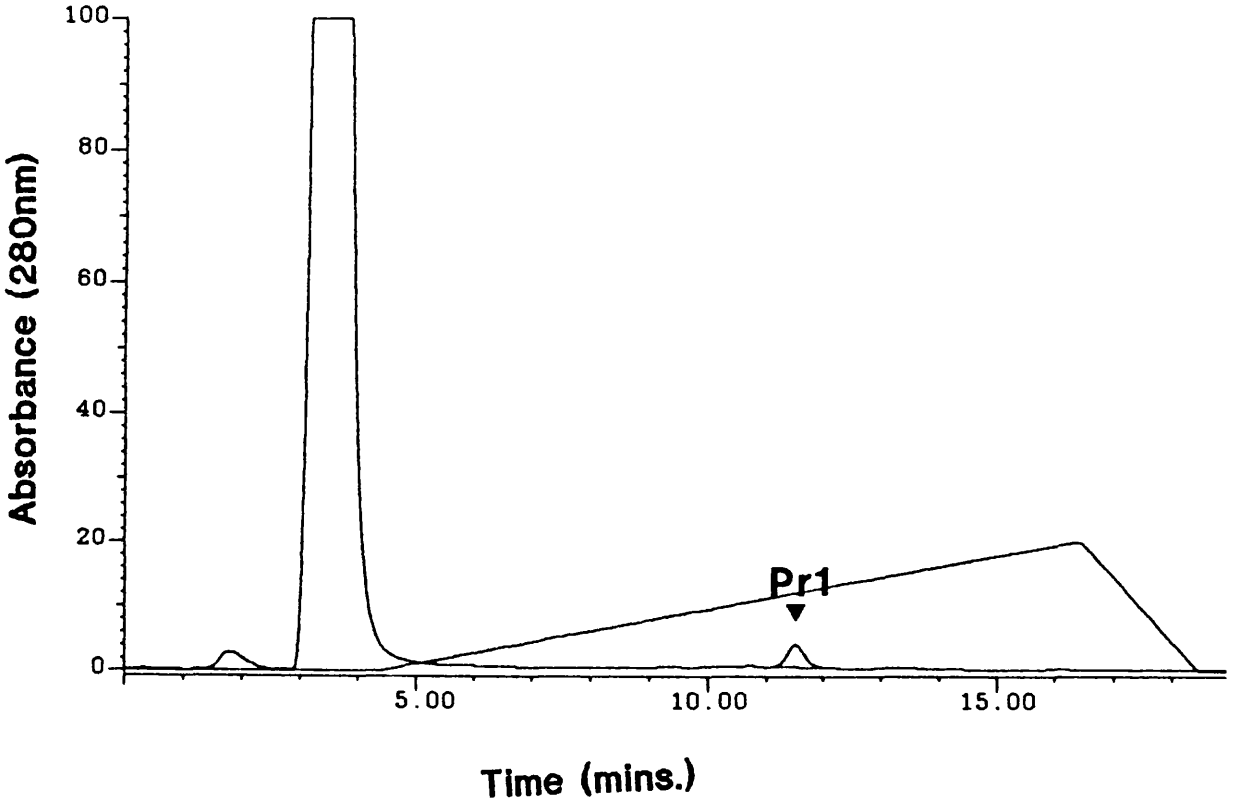
Purification of Pr1 on a 2.4 x 90 cm column of Sephadex G100



Protein was determined by absorption at 280 nm. One unit of activity liberates 1 μmol NA $min^{-1}\mu l^{-1}$ from Suc-$(Ala)_2$-Pro-Phe-NA.

Figure 15

Purification of Pr1 by high-performance cation exchange chromatography



Protein was detected by absorption at 280 nm. Pr1 was assayed as described in the text. The system utilised a Dynamex SCX-Ti (Rainin) column (4.6 mm x 10 cm) equilibrated in 20 mM sodium acetate, pH 5.5. Pr1 was eluted with a linear salt gradient as described in the text.

resulting yield was ca. 3-fold higher than that described by St. Leger et al. (1987a). The concentrated filtrate was first dialysed overnight against dH_2O (pH 6) and then Tris.HCl pH 8 added to give 10 mM. Separation was performed on a Q-Sepharose column (10 x 1 cm) equilibrated in 10 mM Tris.HCl pH 8. Elution of Pr2 was achieved with a linear gradient of 0.01-0.75 M Tris.HCl pH 8 (Fig. 16). To remove all traces of Tris.HCl the protein was TCA precipitated as described in section 2.3.6 prior to sequencing.

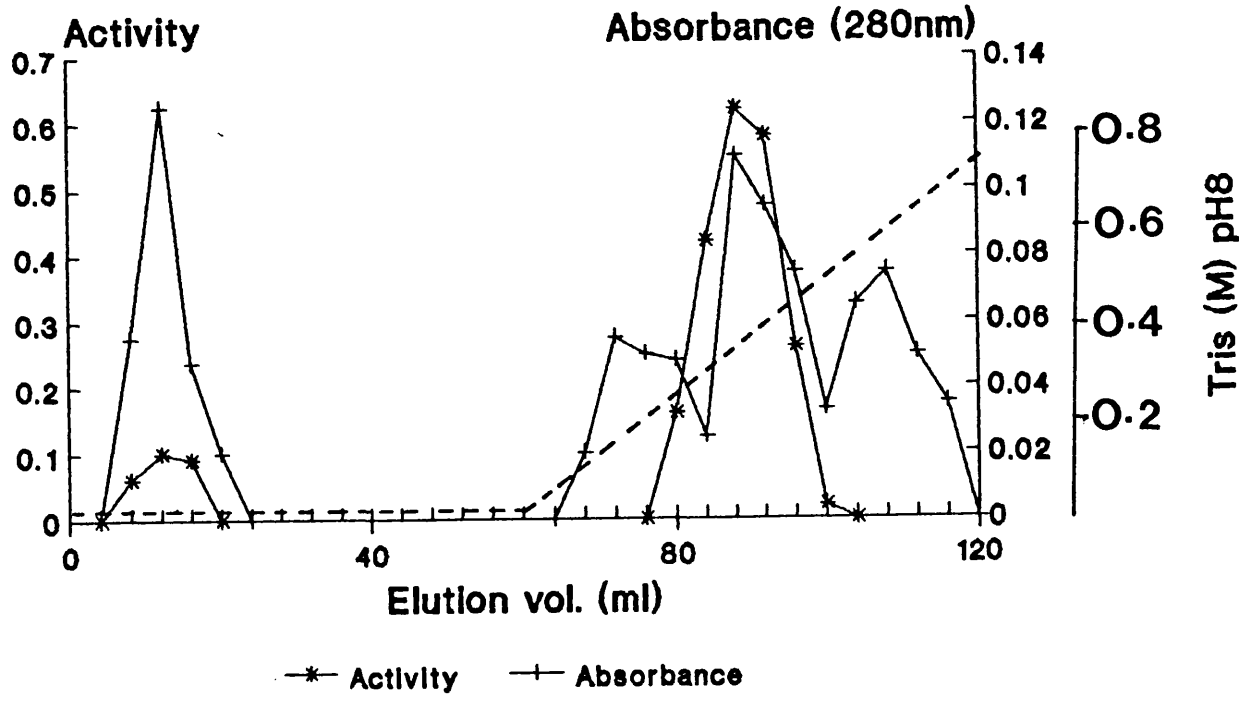
Table 12 summarises the purification schemes used to purify Pr1 and Pr2. The final preparations of Pr1 and Pr2 produced single bands on SDS-PAGE stained with Coomassie blue, but several minor contaminating bands were seen in the Pr1 preparation purified by IEF when the gel was subsequently stained with silver as described in section 2.3.7.

4.1.2. Specificity and titre of antibodies vs Pr1.

In an attempt to generate an antibody probe, to be used for screening a cDNA expression library for Pr1 clones polyclonal antibodies against Pr1 were raised and the IgG component purified as described in section 2.4.5. The titre and specificity of the antibody was tested by Ouchterlony double immunodiffusion (see section 2.4.6) which showed that antibody dilutions up to 1:128 (1:32 before purification) gave precipitin lines against purified, denatured Pr1. Denatured protein was used because

Figure 16

Purification of Pr2 on a 1 x 10 cm column of Q-Sepharose



Protein was determined by absorption at 280 nm . One unit of activity liberates 1 μmol NA $min^{-1}\mu l^{-1}$ from Bz-Phe-Val-Arg-NA. The column was equilibrated and subsequently eluted with a linear Tris gradient (- - -) as described in the text.

Table 12

Purification of Pr1 and Pr2 from *Metarhizium anisopliae*

Purification	Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Yield (%)	Purification (-fold)
A) Pr1-IEF					
Crude extract	79	4281	54.2	100	1
Preparative IEF (pH 3-10)	33	3978	120.5	93	2.2
Sephadex G-100	12	2362	196.8	55	3.6
B) Pr1-HPLC					
Crude extract	67	3726	55.6	100	1
Cation exchange	14	2725	194.7	75	3.5
C) Pr2					
Crude extract	32	1430	44.7	100	1
Q-Sepharose	3	447	149	31	3.3

Chromatographic and assay procedures are described in the text. One unit of activity liberates 1 μmole of nitroanilide from Suc-$(Ala)_2$-Pro-Phe-NA (Pr1) and Bz-Phe-Val-Arg-NA (Pr2) per minute at pH 8 and 25°C.

recombinant protein produced by the lambda gt11 expression library would not be post translationally modified. The antibody also cross-reacted with denatured Pr2 and Pr4 (supplied by Dr. S. Cole) at a dilution of 1:64 an effect not reported for the antibodies produced in a similar way by St. Leger et al. (1987c).

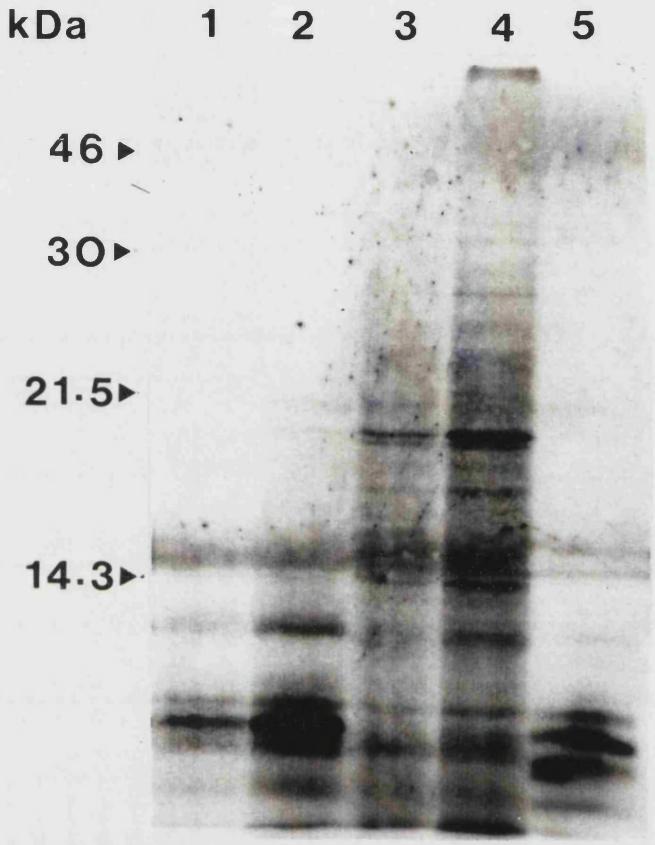
4.1.3. Construction and screening of a cDNA library

The cDNA library was constructed before the protease regulation studies were carried out, so in an attempt to maximise the possibility of isolating Pr1 clones RNA was extracted from mycelium in liquid cultures of basal salts medium containing 1% ground cuticle (see section 4.1.9), inoculated with 10^7 conidia and incubated for 3-7 days as described in section 2.2.1. Protease levels were rising during this time suggesting the presence of protease specific mRNA within the cells.

The mRNA was isolated on mAP as described in section 2.4.3 and then translated <u>in vitro</u> (section 2.4.4) to test the integrity of the mRNA and to determine if any proteins with a molecular weight similar to Pr1 (≈30,000)and Pr2 (≈29,000) were expressed. The products were separated by SDS-PAGE and detected by autoradiography (Fig. 17). The translations showed that the mRNA from 3 and 4 day cultures was of sufficient quality to translate proteins, but no proteins of a high molecular weight were translated and no proteins with a molecular weight of 25-35 appeared to be highly expressed. Examination of the translations

Figure 17

In vitro translation of poly (A^+) RNA from *Metarhizium anisopliae* grown with insect cuticle as sole carbon and nitrogen source



Poly (A^+) RNA was extracted from mycelia incubated with cuticle for 6 days (lane 1), 5 days (lane 2), 4 days (lane 3) and 3 days (lane 4) was translated in the presence of ^{35}S methionine and the proteins separated on a 10% acrylamide gel. A water control is included (lane 5). The products were visualised by autoradiography as described in the text.

by immunoprecipitation (see section 2.4.6) did not reveal the presence of any *in vitro* translated Pr1, but the low titre and cross reactivity of the antibody might explain this.

The mRNA from 3 and 4 day cultures was pooled and a cDNA library constructed as described in section 2.4.11. The titre of the library was 1.2 X 10^4 and contained approximately 50% non-recombinant phage. A library of 10^7 containing less than 5% non-recombinants should be obtainable with this system if the starting RNA is of good quality. The reason for the low number of recombinants is not known, but could be explained by a partial degradation of the RNA (see section 4.1.9).

Immunoscreening the cDNA library:

On dot blots of denatured protein an antibody dilution of 1:50 gave the highest sensitivity and lowest background with the screening protocol to be used for screening the library . Use of the antibody at this dilution facilitated detection of 20 ng of denatured Pr1 spotted on to nitrocellulose.

The entire contents of the cDNA library was screened with the antibody at a dilution of 1:50 as described in section 2.4.12, but no positive clones were isolated.

4.1.4. Genomic library construction.

A library of genomic DNA fragments from M. anisopliae was constructed as described in section 2.4.3 Plating the library on both LE 392 and P2 392 cells gave a titre of 2 X 10^5 phage suggesting that the number of non-recombinant phage in the library was negligible. After amplification the titre of the final stock was 10^{10} pfu ml^{-1}. Analysis of a random sample of clones revealed an insert size of 13-15 kb (eg. Fig. 18.).

Assuming that the genome size of Metarhizium is similar to the ca. 3-4 X 10^7 bp of A. nidulans (Timberlake, 1978) and N. crassa (Krumlauf and Marzluf, 1979), then the size of library required in order to have a 99% probability of having cloned a particular DNA sequence can be estimated with the following equation (Clarke and Carbon, 1976):

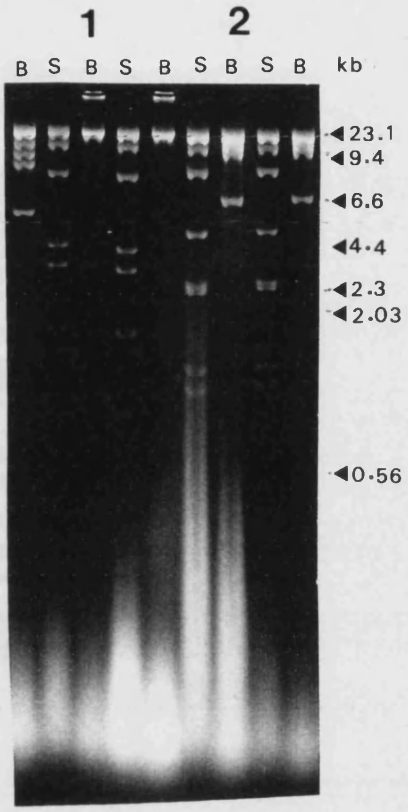
$$N = \frac{\ln(1-p)}{\ln(1-x/y)}$$

If x = insert size, y = genome size (estimated at 4 X 10^7 bp) and making p = 0.99 (a 99% chance of a sequence being represented in the library).

If a conservative estimate of 10 kb is used for the insert size then the pre-amplified library must contain at least 1.8 X 10^4 recombinants. The library made contained 1.2 X 10^5 recombinants and should therefore be a representative library.

Figure 18

Restriction digests of purified clones from the genomic library



Individual plaques were picked and the DNA extracted as described in the text. DNA from two clones was digested with either Sal 1 (S) or Bam H1 (B) and separated on a 1% agarose gel. The DNA was visualised by incorporating ethidium bromide (0.5 μg ml^{-1}) in to the running buffer. The positions of lambda H III molecular size markers are indicated.

4.1.5. Purification of tryptic peptides from Pr1.

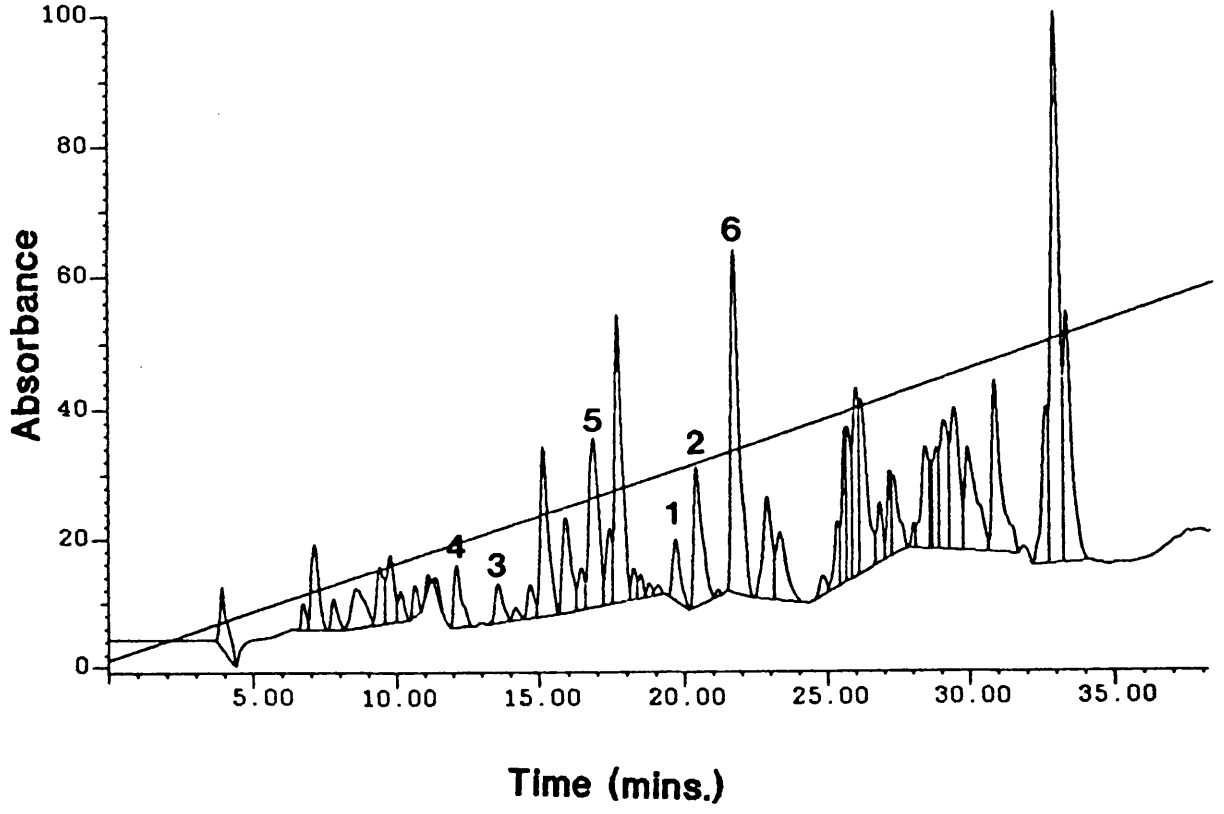
In order to obtain partial internal amino acid sequences of Pr1 the enzyme was digested with bovine trypsin as described in section 2.3.4 and then separated by reverse phase chromatography using a RP318 Hi-pore ODS (Bio-Rad) column. The mobile phase consisted of a linear 0-80% acetonitrile gradient in 0.05% trifluoroacetic acid with detection at dual wavelengths of 220 and 280 nm (Fig. 19). Peaks corresponding to well separated peptides were collected and subjected to another round of purification. Any co-purifying peptides were removed by reverse phase chromatography on the same column with a mobile phase of 0-80% acetonitrile in 20 mM ammonium acetate pH 6. The resulting peptides (eg. Fig. 20) were then sequenced.

4.1.6. Protein sequencing and construction of oligonucleotides.

The primary structure of bovine chymotrypsin, bovine trypsin and porcine elastase are strikingly similar being ca. 40% homologous. These and other related enzymes have a conserved sequence around the active serine of Gly-Asp-Ser-Gly-Gly-Pro (Brenner, 1988). It is therefore likely that Pr1 (chymoelastase) and Pr2 (trypsin) will contain a similar active site sequence to chymotrypsin, trypsin and elastase. Before any amino acid sequence for Pr1 and Pr2 was obtained, the following oligonucleotide corresponding to

Figure 19

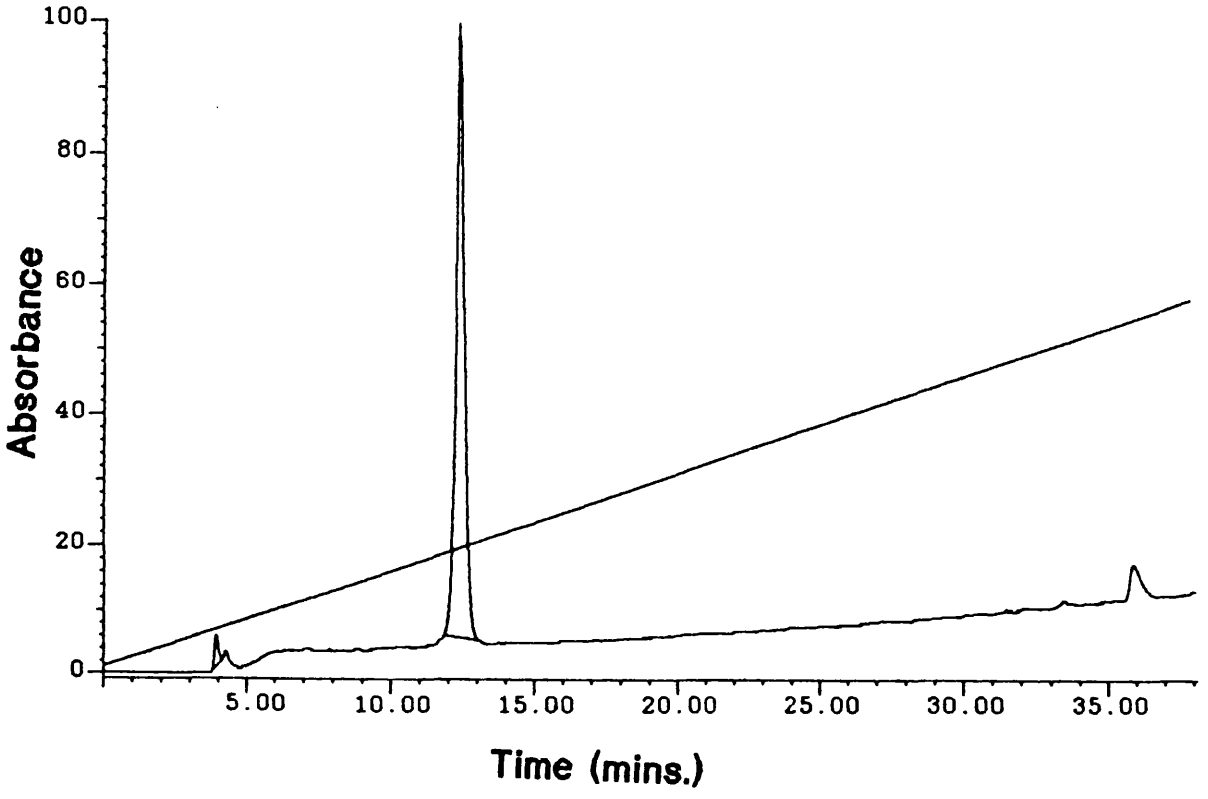
Separation of tryptic peptides of Pr1 by reverse phase HPLC



The peptides were detected by absorption at 220 and 280 nm. The system utilised a RP318 Hi-pore ODS (Bio-Rad) column equilibrated in 0.05% trifluoroacetic acid (TFA). The peptides were eluted using a linear gradient of 0-80% acetonitrile in 0.05% TFA. Numbers correspond to sequenced peptides (1 = peptide 1, etc.)

Figure 20

Purification of tryptic peptides of Pr1



Peptides were separated and detected as described for Fig. 19. Individual peaks were collected and subjected to a further round of purification using a mobile phase of 0-80% acetonitrile in 20 mM ammonium acetate, pH 6.

the trypsin family active site consensus sequence was synthesised:

Serine oligo: 5' GGT-GAT-TCA-GGT-GGT-CC 3'
C C C C C
T
G

Codon usage for the active serine residue was deduced from codons used in other serine proteases (Brenner, 1988). The codon usage for Gly codons in N. crassa and A. nidulans shows that purines are rarely used in the third position (Gurr et al., 1987); so in an attempt to reduce redundancy, pyrimidines were used in the third position in this oligonucleotide, although there is no evidence to suggest that a similar preference exists in Metarhizium (also a filamentous fungus). Screening the genomic library for phage which contain this consensus sequence might isolate clones of serine protease genes of Metarhizium.

The N-terminal sequence of Pr1 could not be determined as the protein proved to be blocked at the N-terminus (see section 2.3.8) which necessitated the purification (as described above) and sequencing of tryptic peptides of Pr1. Initially the amino acid sequences of two peptides were derived:

Peptide 1: Thr-Ser-Phe-Leu-Pro-Tyr-Asn-Asn-His-Leu-Gln-Gln-Met-Lys.

Peptide 2: Met-Ile-Ala-Ser-Gln-Tyr-Pro-Ser-Asn-Val-Lys.

At present no codon usage data exist for Metarhizium which meant that degenerate pools of oligonucleotides and/or "guessmers" had to be synthesised. A degenerate pool of oligonucleotides contains all DNA sequences that can code for a given sequence of amino acids, whereas guessmers' contain only a subset of the possible codons for each amino acid. Guessmers should only be used in cases where long stretches of amino acid sequence is available (sufficient for > 30 nucleotides) so the detrimental effect of mismatches are outweighed by the increased stability of hybrids formed by longer oligonucleotides (Sambrook et al., 1989).

For peptide 1 the following 3 oligonucleoties were synthesised avoiding the serine and leucine (each encoded by 6 possible codons)in the first 4 residues:

Oligo 1: 5' CCT-TAC-AAC-AAC-CAT-TTT-CAG-CAG-ATG-AAG 3' 30-MER

Oligo 1a: 5' TAT-AAT-AAT-CAT-CTA-CAA-CAA-ATG-AA 3' 27-mer

```
              C   C   C   C   T   G   G
                              C
                              G
```

Oligo 1b: 5' TAT-AAT-AAT-CAT-TTA-CAA-CAA-ATG-AA 3' 27 mer

```
              C   C   C   C   G   G   G
```

For peptide 2 only a single oligonucleotide (guessmer) was synthesised as the occurrence of two internal serine residues precluded the use of degenerate pools of oligonucleotides.

Oligo 2: 5' ATG-ATC-GCT-TCT-CAG-TAT-CCT-TCT-AAT-GTT-AAG 3' 33-mer

No positively hybridising plaques were identified from the

genomic library using these oligonucleotides necessitated the purification and sequencing of more peptides as follows:

Peptide 3: Asp-Tyr-Val-Ala-Gln-Asp.

Peptide 4: Leu-Tyr.

Peptide 5: Gly-Ile-Thr-Glu-Gln-Ser-Gly-Ala-Pro.

Peptide 6: Ile-Ile-Asp-Thr-Gly-Ile-Glu-Ala-Ser-His-Pro-Glu-Phe-Glu-Gly-Arg.

The following 44-mer non-degenerate oligonucleotide coding for peptide 6 was synthesised:

Oligo 3:

5' ATT-ATT-GAT-ACT-GGT-ATT-GAG-GCT-TGT-CAT-CCT-GAG-TTT-GAG-GG 3'

The intercodon C-G occurs at about half the expected frequency (Lathe, 1985), so to avoid the possibility of this intercodon T was the prefered base in the third position wherever relevant. Also, there is some evidence that dG:dT base pairs are more stable than dC:dG base pairs (Martin and Castro, 1985) which reinforced the choice of T in third positions and also suggested that G should be used as opposed to A for glutamic acid.

The first 20 residues from the N-terminus of Pr2 were also sequenced and shown to be:

Ile-Val-Gly-Gly-Glu-Ala-Ala-Ala-Gln-Gly-Glu-Phe-Pro-Tyr-Ile-Val-Ala-Leu-Leu-Ser

This sequence contains many amino acids with 3 or more possible codons, therefore to reduce the effects of mismatching, a 44-mer oligonucleotide corresponding to the first 15 residues was synthesised with the 'neutral' base inosine (I) incorporated into

the third position of all codons as follows:

Pr2 oligo: 5' ATI-GTI-GGI-GGI-GAI-GCI-GCI-GCI-CAI-GGI-GAI-TTI-CCI-TAI-AT 3'

Inosine forms a stable base pair with all four conventional bases (Martin and Castro, 1985) and probes containing inosine have been shown to be useful for cloning genes for proteins containing degenerate codons (Ohtsuka et al., 1985).

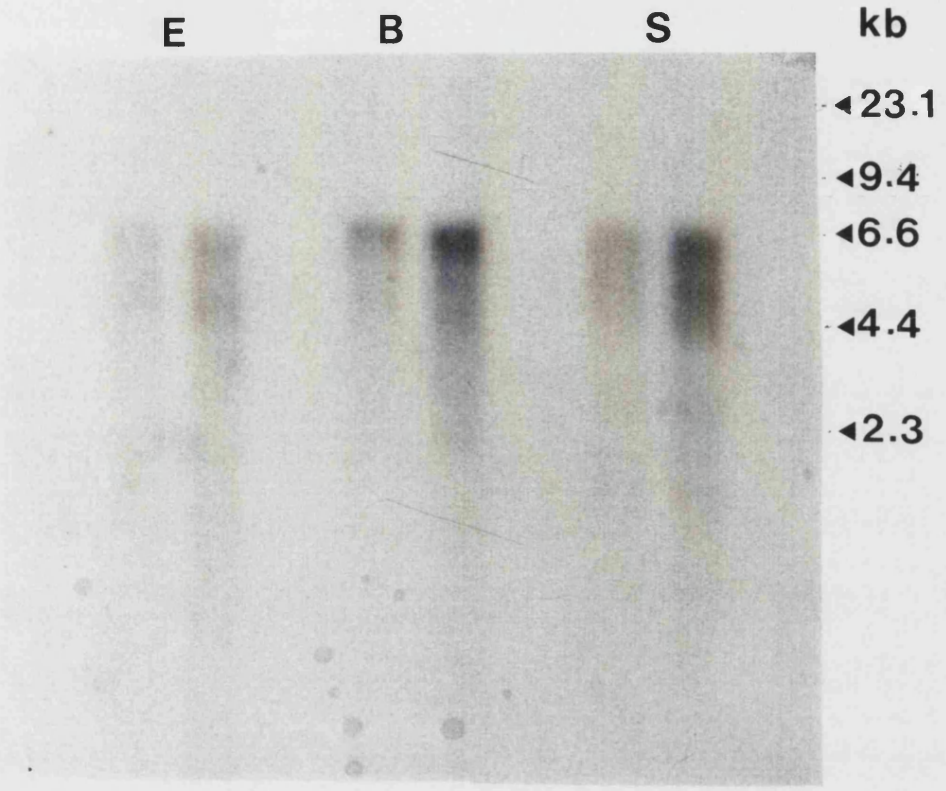
4.1.7. Southern hybridisation of oligonucleotides to genomic DNA.

Any homologous hybridisation seen between Southern blots of genomic DNA and an oligonucleotide could give an indication of the viability of using a particular oligonucleotide to isolate clones from a gene library. The Pr1 oligonucleotides 1 and 2 initially available and the serine oligonucleotide were used as probes against restriction digests of M. anisopliae genomic DNA to look for any homology.

Probing Southern blots of restriction digests of genomic DNA with ^{32}P labelled serine oligonucleotide as described in section 2.4.18 at 37°C showed no homologous hybridisation (Fig. 21). The oligonucleotide binds non-specifically to the DNA with the radioactive signal being proportional to the concentration of the DNA in each track. Hybridisation at a higher stringency resulted in no hybridisation at all.

Figure 21

Southern hybridisation of Metarhizium anisopliae DNA to the consensus serine oligonucleotide



Approximately 1μg of DNA digested with Eco R1 (E), Bam H1 (B) or Sal 1 (S) was separated on a 0.8% agarose gel, transferred to a nylon membrane (Hybond-N, Amersham Int.) and probed with the ^{32}P-labelled serine oligonucleotide. Hybridisation was carried out at low stringency (37°C in 5x SSC, washed 2 x 15 mins. 5x SSC 37°C) and the membrane then subjected to autoradiography as described in the text. The positions of lambda Hind III molecular size markers are indicated.

When similar blots were probed with the Pr1 oligonucleotide 1 at 45°C some specific hybridisation was observed (Fig. 22) suggesting that the oligonucleotides could be used to isolate clones from the genomic library. Oligonucleotide 2 did not hybridise to a similar blot.

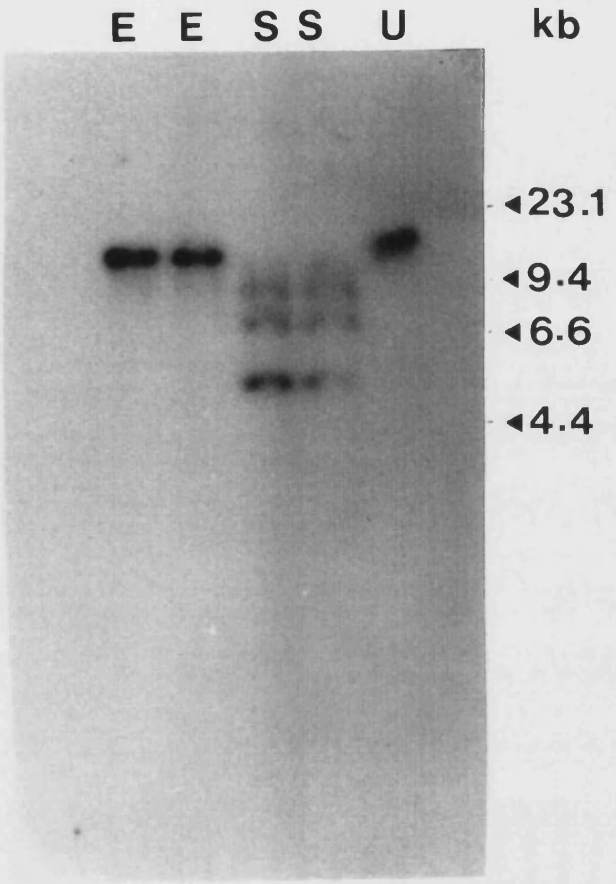
Similar experiments by other workers in this laboratory has shown that genes can be isolated from DNA libraries with oligonucleotides which fail to hybridise specifically to southern blots of genomic DNA (J.M. Clarkson, pers. comm.), possibly due to the higher concentration of target sequences in phage plaques than in southern blots. As a result the serine oligonucleotide was still used to screen the genomic library and as time was limiting the 44-mer Pr1 oligo 3 and the 44-mer Pr2 oligonucleotide were used without prior testing on Southern blots.

4.1.8. Screening the genomic library with synthetic oligonucleotide probes.

The library was screened with the oligonucleotides described in section 3.2.4 in an attempt to isolate protease specific genomic clones. In a typical experiment 40,000 recombinants (plated and lifted as described in sections 2.4.10 and 2.4.15) were screened with 20-100 pmoles of ^{32}P-labelled oligonucleotide in 100 ml of hybridisation solution. The number of recombinants screened in each experiment was at least twice the number required to screen

Figure 22

Southern hybridisation of Metarhizium anisopliae DNA to the Pr1 oligonucleotide 1



Approximately 1 µg of DNA either undigested (U) or digested with Eco R1 (E) or Sal 1 (S) was separated on a 0.8% agarose gel, transferred to a nylon membrane (Hybond-N, Amarsham Int.) and probed with the ^{32}P-labelled oligonucleotide 1. Hybridisation was carried out at low stringency (45°C in 5x SSC, washed 2x 15 mins. 5x SSc, 37°C) and the membrane subjected to autoradiography as described in the text. The positions of lambda Hind III molecular size markers is indicated

representative library (see section 3.2.5). A variety of conditions were used to screen the library with the oligonucleotides initially available (oligos. 1, 1a, 1b, 2 and serine). In an attempt to reduce the effects of potential mismatches the solvent tetramethyl ammonium chloride (Me_4NCl) was used in some cases. The T_m of hybrids in this solvent is independent of its base composition and dependent primarily on its length (Wood et al., 1985). The range of conditions used to screen the library with these oligonucleotides and the conditions used to isolate clones with oligo 3. and the Pr2 oligo. are given in Table 13.

No positive clones were isolated with oligos. 1, 1a, 1b, 2 and serine. Any plaques which appeared to be positive after the first screen were picked, re-plated and screened as before, but all putative positives failed to hybridise in the second round of screening.

Putative positive clones were subsequently picked with Pr1 oligo. 3 and the Pr2 oligo. The temperature of the stringency wash required to isolate positive clones was deduced from a series of pilot experiments with washes performed at a range of temperatures. Washing at too low a temperature resulted in the oligonucleotide hybridising non-specifically to the majority of plaques (Fig. 23) and at too high a temperature no hybridisation was observed at all. The putative positives (4 for Pr1 and 10 for Pr2) were picked by spotting the plaque onto a fresh plate of

Table 13

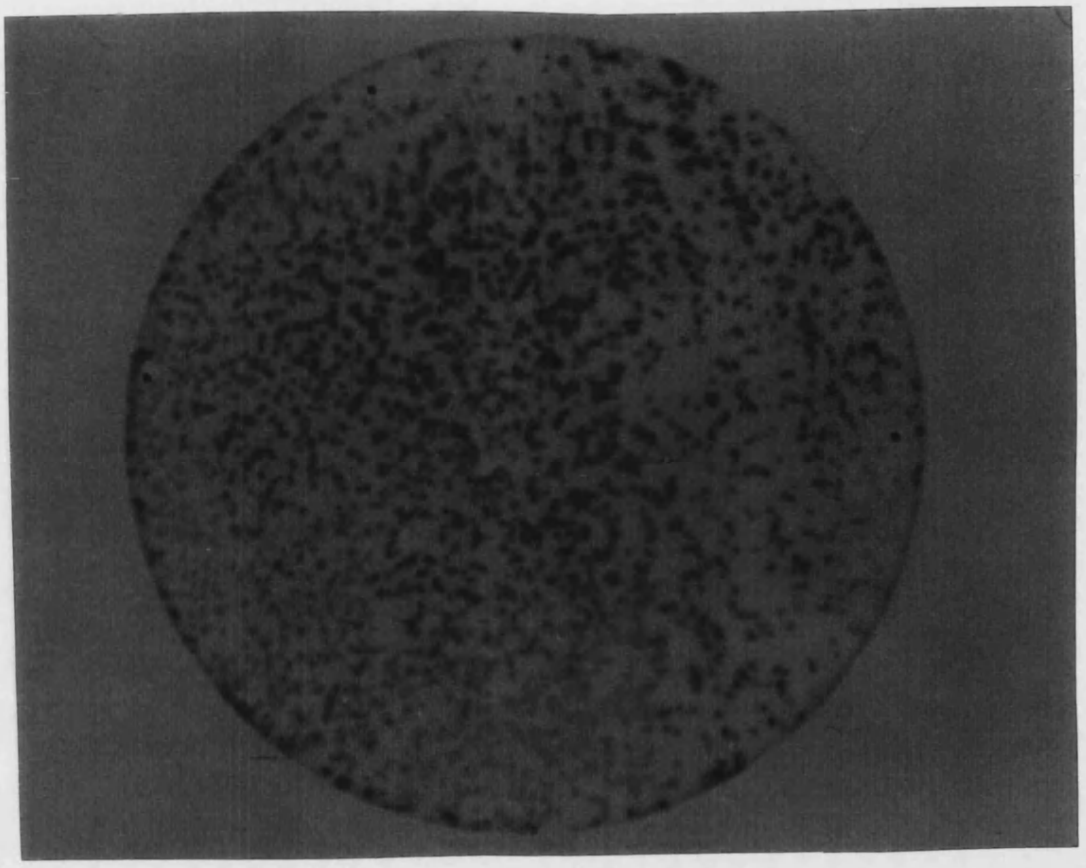
Hybridisation and stringency conditions used to screen the genomic library with synthetic oligonucleotides

Hybridisation conditions	Pre-hyb. Temp.°C	Hyb. Temp.°C	Wash conditions
1) Oligos. 1, 1a, 1b, 2 and serine.			
5X SSC, 4X Denhardt's, 7% SDS, 50 mM Na phosphate pH 7.0.	37	37	5X SSC, 0.1% SDS 37 or 45°C
	45	45	
	65	45	
6X SSC, 4X Denhardt's 1% SDS, 50 mM Na phosphate pH 7.0.	42	42	6X SSC, 0.1% SDS, 42°C or 3 M Me_4NCl, 25 mM Tris.HCl pH 8, 0.02 M EDTA 55°C oligo 1, 1a, 1b. 50°C oligo 2
6X SSC, 4X Denhardt's, 1% SDS, 0.05% Na pyrophosphate, 100 $\mu g\ ml^{-1}$ ss DNA.	37	37	5X SSC, 0.05% Na pyrophosphate, 37°C. or 3 M Me_4NCl, 25 mM Tris.HCl pH 8, 0.02 M EDTA. 55°C oligo 1, 1a, 1b. 50°C oligo 2.
2) Oligos. 3 and Pr2.			
6X SSC, 4X Denhardt's, 1% SDS, 0.05% Na pyrophosphate, 100 $\mu g\ ml^{-1}$ ss DNA.	37	37	1X SSC, 0.05% Na pyrophosphate. 50°C oligo 3 55°C Pr2 oligo 1a, 1b. 50°C oligo 2

Conditions used to screen ca. 40,000 recombinants per experiment from the genomic library as described in the text.

Figure 23

Screening of the lambda genomic library with the Pr1 oligonucleotide 3 at low stringency



The genomic library was plated and the plaques immobilised on nitrocellulose as described in the text. The filter was probed at low stringency (5x SSc, 45°C; washed 2x 15 mins. 5x SSc, 45°C) with the ^{32}P-labelled oligonucleotide 3 and then subjected to autoradiography as described in the text.

cells as described in section 2.4.20 and screened as before. All the clones subsequently hybridised after a second round of screening (eg. Fig. 24). All 4 clones of Pr1 hybridised well and 4 of the 10 putative Pr2 clones also hybridised strongly, suggesting they were indeed the clones of interest. Time did not permit further confirmation of this, which could be achieved by further probing with other specific oligonucleotides or ultimately by sub-cloning and sequencing of the clones to try and identify the amino acid sequences deduced from the partial protein sequences described in section 4.1.6.

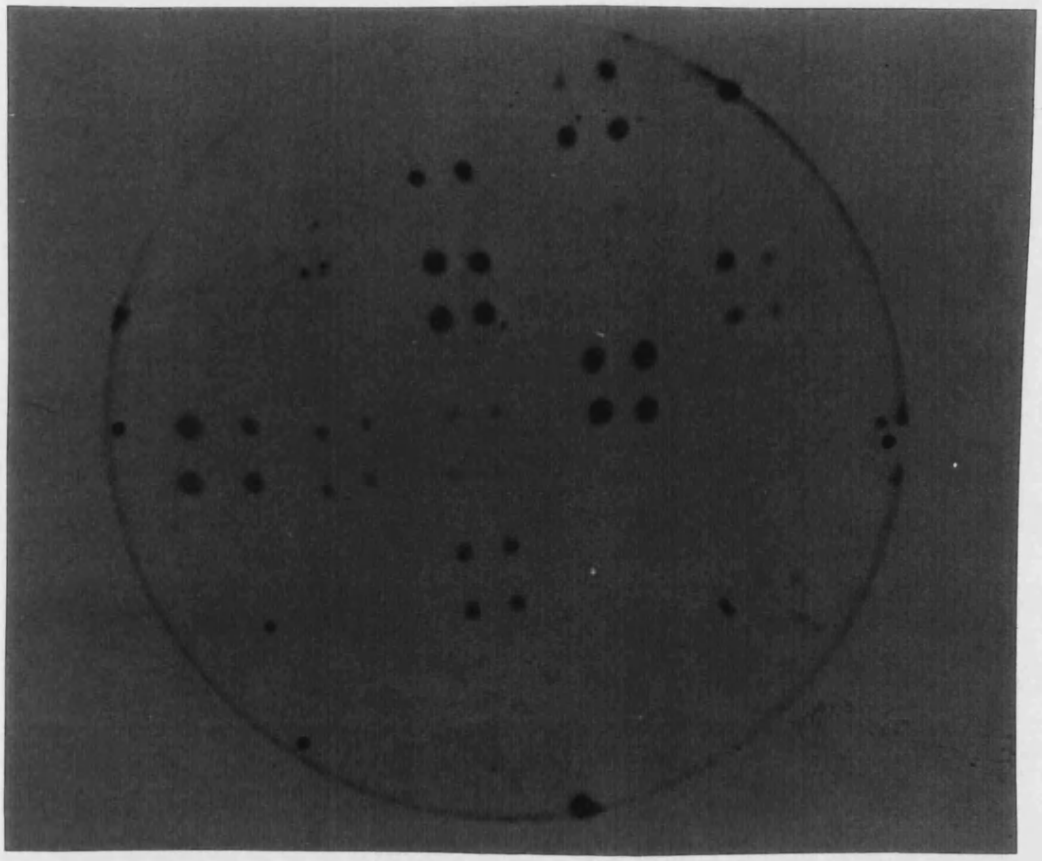
4.1.9. RNA extraction from derepressed and induced mycelium.

Studies of protease regulation in Metarhizium (section 3.1) have shown that high levels of extracellular Pr1 and Pr2 can be achieved by the addition of insect cuticle to liquid cultures starved of carbon and nitrogen for 24 hrs. Following the addition of the cuticle, protease levels rise rapidly suggesting that the cells may contain relatively high amounts of protease specific mRNA. RNA extracted from such cultures could be used for cDNA synthesis and subsequent cloning of protease genes.

Cultures were grown in complete medium for 3-days, starved of C and N for 24 hrs, induced as described and mycelial RNA extracted from cultures at different times during the induction of protease. Analysis of the RNA by agarose gel electrophoresis showed considerable degradation of the extractable RNA in

Figure 24

Hybridisation of putative positive plaques to the Pr2 oligonucleotide



Positively hybridising plaques, plus adjacent colonies from a first round screen were selected with a tooth pick and transferred to nitrocellulose as described in the text. The hybridisation was carried out at moderately high stringency (55°C, washed 2x 15 mins. 1x SSC) and the filter subjected to autoradiography as described in the text.

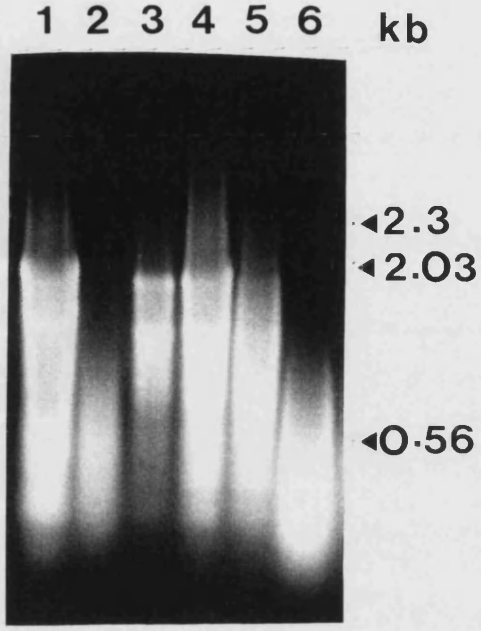
cultures starved of C and N for extended periods (> 12 hrs) and in cultures subsequently induced with cuticle (Fig. 25). Repeated attempts to extract good quality RNA from induced mycelium all resulted in degraded RNA; this could account for the low titre of the cDNA library, as the mycelium was grown in non-repressing inducing conditions. St. Leger et al. (1991b) reported a similar problem of RNA degredation in starved cultures when using a different RNA extraction protocol utilising guanidine.HCl.

4.1.10. Generation of a Pr1 gene fragment using the polymerase chain reaction.

Several methods have been described using the polymerase chain reaction (PCR) to generate fragments of cDNAs. If the amino acid sequence of two regions of a gene is known then the region of cDNA between the two sequences can be amplified using oligonucleotide pools corresponding to the two amino acid sequences using a technique known as MOPAC (mixed oligonucleotide primed amplification of cDNA) as described by Lee et al. (1988). A variation of this technique called rapid amplification of cDNA ends (RACE) was described by Frohman et al. (1988); subsequently, a more specific method known as RACE using nested primers has been described (Frohman and Martin, 1989). In this technique first strand cDNA is primed using a synthetic oligonucleotide containing a dT-tail and a sequence of non-specific nucleotides, usually containing restriction sites. Two oligonucleotides corresponding to regions of the non-specific DNA within the

Figure 25

RNA extraction from derepressed and induced cultures



RNA was extracted from mycelia grown in CM (lane 1), starved of C and N for 24 hrs (lane 2), 6 hrs (lane 3), 9 hrs (lane 4), 12 hrs (lane 5) or 12 hrs after the addition of cuticle (1%) to cultures starved of C and N for 24 hrs (lane 6) and separated on a 1.2% agarose gel. The RNA was visualised by incorporating ethidium bromide (0.5 μg ml^{-1}) in to the running buffer.

primer are used to prime amplification of the cDNA of interest with two overlapping gene specific primers complementary to a region of amino acid sequence. The two 'outer' primers being used to amplify the gene from the cDNA pool and the two 'inner' oligonucleotides priming amplification of the gene from the products of the first round amplification.

This technique was used to generate a Pr1 gene fragment as follows: Mycelium from 3 day CM cultures was transferred to buffered basal salts medium as described previously (see sections 2.2.1 and 2.2.2) and incubated until Pr1 and Pr2 activity was just detectable in culture filtrates (20-25 [Pr1], 35-40 [Pr2] nmols NA $ml^{-1}min^{-1}$). RNA was then extracted from the mycelium and mRNA purified using a poly(A) Quik column (Stratagene) as described in sections 2.4.2 and 2.4.3. First strand cDNA was synthesised as described in section 2.4.10 using the following primer:

5' GACTACGTTAGCATCTAGAATTCTCGAG-$(dT)_{17}$3'

The two 3' primers, R_o and R_i, were:

5' GACTACGTTAGCATCTA 3' - R_o

R_i- 5' CATCTAGAATTCTCGAG 3'

The two Pr1 gene specific 5' primers were derived from the Pr1 44-mer oligo 3 as follows:

5' ATTATTGATACTGGTATTGAGGCTTGTCATCCTGAGTTTGAGGG 3'- 44 MER

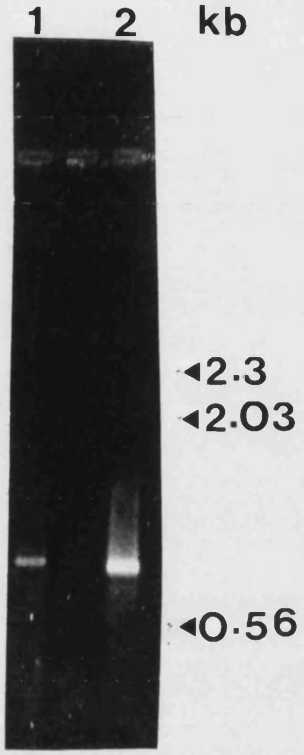
5' ATTATTGATACTGGTATTGAGGCT - $gene_o$

$gene_i$ - 5' CATCCTGAGTTTGAGGG 3'

The Pr1 fragment was first amplified by PCR using the outer primers R_o and $gene_o$ using the following protocol:
In a 0.75 ml microfuge tube 5 µl cDNA solution (diluted to 1 ml in TE [10 mM Tris.HCl pH 7.5, 1 mM EDTA]) was mixed with 1 µl 20X reaction buffer (1 M Tris.HCl pH 9, 400 mM ammonium sulphate, 30 mM $MgCl_2$), 1 µl 4 mM solution of each dNTP, 1 µl 20 µM 5' primer ($gene_o$), 1 µl 20 µM 3' primer (R_o), 10.5 µl dH_2O and 0.5 µl replinase. The reaction was overlaid with 20 µl of sterile mineral oil and thermocycled as follows:
Step 1 94°C 1 min. - Denaturation
Step 2 94°C 1 min. - Cycled denaturation
Step 3 45°C 1 min. - Annealing
Step 4 72°C 1 min. - Elongation
Step 5 72°C 15 mins. - Final elongation
Steps 2-4 were repeated 45 times and the products then diluted 1:20 with TE. Then, 1 µl of the diluted products were amplified as described above with the 5' primer $gene_I$ and the 3' primer R_I with the annealing temperature reduced to 40°C. The products of each reaction were analysed by agarose gel electrophoresis and revealed a single fragment was amplified in each reaction (Fig. 26). Confirmation that the amplified DNA was a fragment of the Pr1 gene was achieved by sequencing as described in the following section.

Figure 26

Amplification of a cDNA fragment by PCR using Pr1 specific oligonucleotide primers



A cDNA fragment was amplified using Pr1 gene specific primers as described in the text. The products of the first round of amplification (lane 1) and the second round (lane 2) were separated on a 1% agarose gel and visualised by incorporating ethidium bromide (0.5 μg ml^{-1}) in to the running buffer.

4.1.11. Sequencing of the PCR amplified DNA fragment.

The products of a first round amplification as described above were separated by electrophoresis on a 1% agarose gel, the single predominant band excised and the DNA extracted and purified using Geneclean. The DNA was resuspended in 20 μl dH_2O, 7 μl of which was sequenced in one direction as described in section 2.4.23 using $gene_D$ as the primer.

The short DNA sequence and derived amino acid sequence deduced from two gel runs is given in Fig. 27.

Translation of the DNA sequence revealed two possible open reading frames with two internal stop codons appearing within the third sequence. The amino acid sequence of ORF 3 contains the sequences Asp-Tyr-Val-Ala-Gln-Asp and Leu-Tyr which correspond to the amino acid sequences of the Pr1 peptides 3 and 4 respectively. It is not possible to deduce which of the open reading frames is translated, but the amplification protocol is designed to amplify specific cDNAs and the homologous sequences within ORF 3 strongly suggests that the cDNA fragment amplified represents part of the Pr1 gene.

Time did not permit the complete sequencing of the fragment which could confirm the translated reading frame and may identify other known peptide sequences which would confirm that the amplified cDNA is a fragment of the Pr1 gene.

Figure 27

Partial DNA sequence of the PCR amplified cDNA fragment

a) DNA sequence

CCAAAAAGGCTAAGCTCTATGGTGTCAAGGTTCTTGACAACCAGGGCAGTGGTTCC

TACTCCGGTATCATCAGTGGCATGGACTACGTTGCACAGGACTCCAAGACCCGCG

b) Derived protein sequences:

ORF 1 - Pro-Lys-Arg-Leu-Ser-Ser-Met-Val-Ser-Arg-Phe-Leu-Thr-Thr-Arg-Ala-Val-Val-Pro-Thr-Pro-Val-Ser-Ser-Val-Ala-Trp-Thr-Thr-Leu-His-Arg-Thr-Pro-Arg-Pro-Ala

ORF 2 - Gln-Lys-Gly-STOP-Pro-Leu-Trp-Cys-Gln-Gly-Ser-STOP-Gln-Pro-Gly-Gln-Trp-Phe-Leu-Leu-Arg-Tyr-His-Gln-Trp-His-Gly-Leu-Arg-Cys-Thr-Gly-Leu-Gln-Asp-Pro

ORF 3 - Lys-Lys-Ala-Lys-Leu-Tyr-Gly-Val-Lys-Val-Leu-Asp-Asn-Gln-Gly-Ser-Gly-Ser-Tyr-Ser-Gly-Ile-Ile-Ser-Gly-Met-Asp-Tyr-Val-Ala-Gln-Asp-Ser-Lys-Thr-Arg

Partial DNA sequence (a) of the cDNA fragment amplified using Pr1 specific primers and deduced amino acid sequence (b) of the three open reading frames. Underlined sequences in ORF 3 correspond to peptide sequences obtained from purified Pr1.

4.2. DISCUSSION

In a previous study, St. Leger et al. (1987a) purified Pr1 by wide range IEF followed by affinity chromatography on a column of soya bean trypsin inhibitor-Sepharose. Pr2 was similarly purified, but with an additional narrow range IEF step prior to affinity chromatography. In this study a much improved purification protocol was used for both enzymes. After concentration of culture filtrates, Pr1 was purified to homogeneity using a single step procedure (cation exchange HPLC) resulting in a 3.5-fold purification and 75% yield of the enzyme. Pr2 was also purified using a single step procedure (anion exchange chromatography) resulting in a 3.3-fold purification but only a 31% yield of the enzyme. Collecting a greater number of fractions containing Pr2 activity from the Q-Sepharose column followed by an additional purification step such as affinity chromatography on STI-Sepharose as described by St. Leger et al. (1987a) would be required to obtain a greater yield of purified Pr2. However, the ease with which large quantities of both enzymes can be obtained probably makes this unnecessary. Both purified enzymes gave single bands on SDS-PAGE and therefore were proved to be sufficiently pure for sequencing.

St. Leger et al. (1987a) reported that narrow range IEF separated Pr2 into three isoenzymes with pIs of 4.0, 4.2 and 4.42. In another study in this laboratory no evidence of the three isoenzymes was found and the pI of Pr2 was estimated to be 5.4

(S. Cole, unpublished observations). In this study only a single peak of Pr2 activity was detected after ion exchange chromatography and only one N-terminal amino acid sequence was present in the purified preparation, suggesting that Pr2 exists as single enzyme. It is possible, however, that three isoenzymes exist, but that these co-purified and had the same N-terminal amino acid sequence.

The range of hybridisation and stringency conditions used to screen the genomic library suggests that the failure to isolate clones with the serine protease oligonucleotide and the Pr1 oligonucleotides 1, 1a, 1b and 2 could only have resulted from a lack of homology between the oligonucleotides and the Pr1 gene. This could be due to a number of reasons, namely Pr1 and Pr2 may contain a different active site sequence to the trypsin family of serine proteases; the amino acid sequence of the Pr1 peptides was not accurate (eg. due to an impure sample or a mistake in the sequencing protocol); the translated amino sequences were interrupted in the gene by introns; a too highly degenerate pool of oligonucleotides was synthesised or, in the case of guessmers a high proportion of incorrect nucleotides were used in places of degeneracy. It is not possible to say which of the reasons is the most likely cause of the lack of homology until the entire Pr1 gene has been sequenced when the amino acid sequence and codon usage can be deduced.

Both degenerate pools of short oligonucleotides and longer, less

degenerate oligonucleotides (guessmers) have been used as probes to isolate cDNA and genomic clones. Suggs et al. (1981) synthesised two 15-mer oligonucleotides (8-fold and 32-fold degeneracy) and one 11-mer (8-fold degenerate) deduced from the amino acid sequence of human β_2-microglobulin. cDNA clones were identified with both 15-mers but not with the degenerate 11-mer which suggests that there is a minimum probe length which should be considered when designing oligonucleotides. Small oligonucleotides are likely to hybridise to spurious sequences occurring by chance alone (Lathe, 1985). The human erythropoietin gene was isolated by Lin et al. (1985) from a phage (charon 4A) genomic DNA library using one 20 and one 17-mer oligonucleotide (both 128 fold degenerate) deduced from amino acid sequence. The 20-mer hybridised to 272 phage and the 17-mer to ≈4000, but only 4 clones hybridised to both probes of which 3 contained the correct gene. This demonstrates the necessity of obtaining a number of partial amino acid sequences for a given protein if the identification of a substantial number of false positive clones is to be avoided. At present only the N-terminal twenty residues are known for Pr2, therefore the putative positive clones identified should be further checked for homology to oligonucleotides constructed from internal amino acid sequences (which could derive from the sequencing of peptides as for Pr1) Jacobs et al. (1985) obtained partial sequences of human erythropoetin and constructed three oligonucleotides, a 17 (32-fold degenerate), an 18 (128-fold) and a 14-mer (48-fold) to screen a genomic library. Only two clones isolated hybridised to

all three oligonucleotides. The method of obtaining partial amino acid sequences for a given protein and construction of degenerate probes has been used to clone an aspartate protease (rennin) from the fungus Mucor pusillus. Tonouchi et al. (1986) constructed two 17-mer and two 14-mer probes and screened a cosmid (pjb8) genomic library. One clone hybrised to the 17-mers and one 14-mer; sequencing the clone confirmed its validity by the identifying known peptide sequences. The majority of genes reported to have been cloned with pools of oligonucleotides have been isolated with probes with of a degeneracy of less than 128, although an oligonucleotide pool of 256 has been used to clone a human preproinsulin-like growth factor II gene (Bell et al., 1984). The Pr1 oligonucleotides 1a and 1b have a degeneracy of 256 and 128 respectively which could be at the limit of sensitivity for this approach and could explain the difficulty in isolating clones with these probes. Short degenerate pools of oligonucleotides have also been used as primers in first strand cDNA synthesis reactions which have then been used to isolate clones from gene libraries (eg. Sood et al., 1981). This approach would mean that all hybridisations could be performed using "standard" conditions but any non-homologous priming in the cDNA synthesis reaction would generate non-specific probes.

The use of guesmers was first described by Jaye et al. (1983) who synthesised a single 52-mer oligonucleotide based on the amino acid sequence of bovine factor IX and used it to isolate a cDNA clone of the corresponding human gene. The majority of reported

cases of the use of guessmers for the cloning of genes have utilised probes of at least 40 nucleotides, although the human protein kinase C gene has been cloned with a guessmer of 32 nucleotides (Knopf et al. 1986). Sufficient amino acid sequence to construct non-degenerate probes of at least 30 nucleotides is usually required for this approach (Sambrook et al. 1989). The potential effect of mismatching for the Pr1 oligonucleotides 1 and 2 (30 and 33 nucleotides long respectively) may therefore have been too great if a large number of incorrect nucleotides were used in positions of degeneracy. The effects of such mismatches decrease as the length of the probe increases and could explain why the longer 44-mer probes for Pr1 (oligo 3) and Pr2 identified clones in the genomic library, whereas the shorter probes did not.

The nuclear genes of filamentous fungi can be punctuated by non-coding sequences or introns which occur in about 60% of genes sequenced (Gurr et al., 1987). Of the eukaryotes, fungi have the shortest introns with a mean length of less than 100 nucleotides (Hawkins, 1988). The number of introns in a particular gene is usually less than 3, but there can be as many as 8 and these may be located randomly throughout the gene (Gurr et al., 1987). It is possible that the regions coding for peptides 1 and 2 are interrupted by introns which would explain why genomic clones were not isolated with the corresponding oligonucleotides, if so the probes could be used to isolate genes in a cDNA library.

It is possible that the Pr1 gene does not show any homology to the trypsin family of serine proteases and therefore would not contain the conserved active site amino acid sequence. Members of the trypsin family of proteases have an active serine, a catalytically essential histidine and similar kinetic mechanisms (see Stroud, 1974 for review). This suggests that these enzymes evolved through gene duplications of an ancestral serine protease. Subtilisin also a serine protease bears little sequence homology to chymotrypsin, trypsin or elastase. However, the active site of all these enzymes are essentially identical and their relative 3-D positions are nearly indistinguishable (see Kraut, 1977). Brenner (1988), from a study of active site sequences, suggests that these two lines of proteases convergently evolved from a common ancestor with an active cysteine as opposed to serine. The active site of subtilisin proteases contain a conserved sequence of Gly-Thr-Ser-Met-Ala-ser/Ala/Thr-Pro-His/Phe-Val/Ile which is different from the consensus sequence seen in the trypsin family. Protease A, a serine protease from Streptomyces griseus is a bacterial serine protease with chymotryptic specificity which exhibits some sequence homology and extensive structural similarity to chymotrypsin (James et al., 1980). The ancestral trypsin gene apparently arose before the divergence of prokaryotes and eukaryotes (Brenner, 1988) which implies that the protease genes of Metarhizium could contain either of the conserved active site sequences. The fact that Pr2 is a "classical" trypsin, ie. a serine protease which cleaves peptide bonds on the C-terminal

side of basic amino acids (for a review of serine protease sructure and catalytic mechanisms see Kraut, 1977) and that Pr1 exhibits both chymotyptic and elastase properties (St. Leger et al., 1987b) suggests that the genes are more closely related to the trypsin family of serine proteases. Chymotrypsin rapidly hydrolyses peptide bonds following an aromatic side chain at P_1 (amino acid residues extending from the cleaved bond toward the N-terminus are usually denoted P_1,P_2,P_3 etc.), elastase is less specific but prefers an uncharged nonaromatic residue (eg. alanine) at P_1. The specificity of subtilisin at P_1 most nearly resembles chymotrypsin, but subtilisin will also hydrolyse peptide bonds after amino acids with nonaromatic apolar side chains. Subtilisin is also similar to elastase in preferring extended polypeptide substrates. There are obvious similarities between Pr1 (chymoelastase) and subtilisin, so it is possible that the protease genes of Metarhizium could contain the conserved active site sequence seen in all subtilisin-like enzymes. The conserved sequence in subtilisins is slightly more degenerate than that seen in the trypsin family, making the construction of an oligonucleotide more difficult; a degenerate 15-mer could be constructed from the sequence Gly-Thr-Ser-Met-Ala, but 15-mers are usually considered too small for the screening of complex DNA libraries (Lathe, 1985).

The longer 44-mer oligonucleotides for Pr1 (oligo 3) and Pr2 (see section 3.3.6) successfully isolated clones from the genomic library which gave strong hybridisation signals after two rounds

of screening. The stringency of the post hybridisation washes suggests that the clones are likely to contain sequences similar to the sequence of the oligonucleotides, but unfortunately time did not permit further characterisation of these clones. Ultimate confirmation of the validity of the clones can only come from sub-cloning and sequencing of the hybridising fragments from the clones.

Studies on the regulation of production of Pr1 and Pr2 suggested that RNA extracted from derepressed, cuticle-induced mycelium should contain high levels of protease specific mRNA. However, extended periods of starvation apparently resulted in RNA degradation and precluded the use of protease induction in cDNA cloning stategies. A similar problem was reported by St. Leger et al.(1991b) using a different RNA extraction protocol. In Metarhizium Pr1 transcripts appear 45-60 mins. after transfer of mycelia from nutrient rich media to basal salts (St. Leger et al. 1991b), so RNA extracted from mycelium as soon as Pr1 is detectable in the filtrate should contain Pr1 specific mRNAs. The time of appearance of Pr2 transcripts is not known.

A single fragment of cDNA was amplified using RACE PCR with nested primers (Frohman and Martin, 1989) from a cDNA pool constructed from RNA extracted as described above with primers specific to Pr1. PCR is a powerful method for the in vitro amplification of DNA sequences. Two oligonucleotides, that are complementary to sequences that lie on opposite strands of the

DNA molecule and flank the sequence to be amplified are used in a series of reactions catalysed by a thermostable DNA polymerase (Saiki et al., 1985, 1988). In RACE PCR, cDNA synthesis is primed by oligo (dT) to which another sequence of nucleotides has been added. Two overlapping primers homologous to regions of the additional nucleotide sequence and two non-overlapping gene specific primers are used in two rounds of PCR to amplify gene specific fragments. The second round of amplification uses the pair of internal primers (the two primers closest together) to reduce the possible effects of non-specific amplification or as in the case of Pr1 test whether a single fragment amplified in the first round could be amplified again with a different gene specific primer. This protocol, which is designed to reduce amplification of non-specific products and the identification of two Pr1 peptide sequences (Leu-Tyr and Asp-Tyr-Val-Ala-Gln-Asp) in one of the possible reading frames of the partially sequenced cDNA fragment, confirms that the fragment amplified is a fragment of the Pr1 gene. The 5' region of the cDNA could be isolated by the addition of a d(A) tail to the 5' end of the cDNA with terminal deoxynucleotidyl-transferase (Frohman et al., 1988; Ohara et al., 1989) and amplifying the cDNA using a d(T) primer in conjunction with one of the Pr1 gene specific primers described. This technique has been used to obtain the complete cDNA sequence of a $GABA_{\rho}$ receptor subunit from the mollusc Lymnaea stagnalis (Harvey et al., 1991) and a rhodopsin gene from a lamprey (Hisatomi et al., 1991). Theoretically, a full length cDNA clone of Pr1 could be obtained using PCR with primers

designed from predicted 5'- and 3' untranslated sequences (eg. Harvey et al., 1991). The amplified Pr1 gene fragment could subsequently be used to isolate genomic clones from the library and hybridisation of the amplified fragment to the clones isolated with the Pr1 oligo 3 could confirm the presence of the Pr1 gene in these clones.

The Pr2 oligonucleotide failed to amplify any cDNA when used in similar experiments. This could be due to a lack of Pr2 specific cDNAs in the library which is unlikely because Pr2 is similar to Pr1 in that de novo synthesis of mRNA is involved in enzyme production (St. Leger et al., 1988b) and Pr2 is detectable in culture filtrates before Pr1. A more likely explanation is that because the cDNA was primed with oligo (dT) from the 3' end (as opposed to internally with random hexanucleotides)some cDNAs lack some 5' sequences and the Pr2 oligonucleotide corresponds to the N-terminus of the enzyme, which means that only a full length cDNA would be amplified using this primer. Generation of some internal amino acid sequence might enable the amplification of a Pr2 cDNA fragment.

The method of partial sequencing of a protein followed by gene amplification using PCR use as a gene probe as described for Pr1 would seem to be an efficient way of isolating clones where the protein encoded by the gene can be purified. In cases where there are little or no data for codon usage by a particular organism (eg. Metarhizium) this method could prove to be less time

consuming than screening libraries direct with oligonucleotides and subsequent characterisation of putative positive clones.

The sequence of a cDNA clone of Pr1 has recently been published (St. Leger et al., 1992). The published sequence confirms that the PCR fragment amplified is part of the Pr1 gene and demonstrates that Pr1 shows some homology to the subtilisin family of proteases.

5. GENERAL DISCUSSION

The molecular cloning of protease genes is a prerequisite to a molecular study of their regulation, role in pathogenesis and to any molecular genetic approaches to strain improvement. To that end, putative genomic clones for Pr1 and Pr2 have been identified. In addition, a method has been developed to generate a cDNA fragment of the Pr1 gene which could be used as a probe in other molecular studies.

Understanding the role of cuticle-degrading proteases in cuticle penetration, pathogenesis and virulence is facilitated by studying the regulation of such proteases. This can also synergise with the molecular approach by optimising conditions for the differential synthesis of a protease. The work described here demonstrates conclusively that for at least one strain (ME1) of M. anisopliae the chymoelastase Pr1 is induced by a component(s) of cuticular protein. To the author's knowledge this is the first demonstration of the specific induction of a microbial protease. In common with other fungal proteases, another protease Pr2, a trypsin, was shown to be induced by a range of proteins.

The protease regulation studies described here demonstrate that the three characterised cuticle degrading proteases of Metarhizium Pr1, Pr2 (St. Leger et al., 1987a) and Pr4 (S. Cole, unpublished) are regulated differently. Both Pr1 and Pr2 are

inducible (Pr1 by cuticular protein, Pr2 by a range of proteins), but Pr4 was not detected in any derepressed or induced cultures in this study. Pr4 was originally purified from 6-day cuticle cultures with little activity being detected until day 3 (S. Cole pers. comm.), which together with the fact that Pr4 does not appear to be subject to carbon and nitrogen derepression implies that Pr4 may be produced later in the infection process than Pr1 and Pr2, although the presence of Pr4 during infection has yet to be demonstrated. It is possible that Pr4 is specifically induced by a peptide(s) not encountered until later in infection.

The production by an organism of enzymes with different characteristics (eg. pI, pH optima and mode of action) should confer flexibility under a wide range of conditions. Therefore, it is difficult to speculate as to why Metarhizium needs to produce two proteases with the same substrate specificity and similar pIs and pH optima (Pr2 and Pr4), but it is possible that the fungus produces Pr4 (a cysteine protease) as an alternative protease in response to the inhibition of the two serine proteases Pr1 and Pr2 during infection. Trypsin and α-chymotrypsin inhibitors have been purified from the haemolymph of silkworms (Sasaki, 1978; Eguchi et al., 1982; Sasaki and Kobayashi, 1984). Similar Pr1 inhibitors have been identified in locusts (Gillespie and Charnley pers. comm.), but to date no inhibitors of cysteine proteases have been identified. Pr4 has been shown to exhibit 51% of the cuticle degrading activity of Pr1 (S. Cole, unpublished), so it is likely to be able contribute

significantly to nutrition during infection after the initial cuticle penetration. A study of the regulation of Pr4 similar to the study of Pr1 and Pr2 described here might shed some light on the possible role of this protease. Addition of PMSF to cultures induced with insect cuticle might mimic the inhibition of proteases in insect blood and effect Pr4 production.

In two separate studies Pr2 has been shown to possess 4% (St. Leger et al., 1987a) and 21% (S. Cole, unpublished) of the cuticle-degrading activity of Pr1. Prima facie the role of Pr2 therefore is less obvious than for Pr1, but the present study suggests that Pr2 may play a part in the induction of Pr1. The products of a cuticle digestion with a pure preparation of Pr2 induced Pr1 in derepressed mycelia. Pr2 was detectable in derepressed cultures before Pr1 and it is induced by a range of proteins. This might suggest that during an initial period of saprophytic growth of the fungus Pr2 would be synthesised before Pr1, although it is unlikely that carbon and nitrogen concentrations in the wild would be sufficiently high to completely repress Pr1. Germinating conidia of Metarhizium might initially secrete Pr2 which could be sufficient to maintain saprophytic growth but not growth on insect cuticle, although the cuticle-degrading activity of Pr2 is sufficient to induce Pr1 which has much higher cuticle-degrading activity.

There is some evidence to suggest that Pr2 and Pr4 may have a regulatory role as both enzymes have been shown to be capable of

rapidly activating an inactive zymogen or proenzyme of Pr1 (S. Cole, unpublished). Addition of Pr1 to the proenzyme results in autoactivation but at a considerably slower rate than with the other two enzymes.

The nature of any protease inducing molecule (other than general proteins which could not function as intracellular effector molecules) has never been identified. This seems remarkable in view of the commercial significance of proteases and the many reported studies on their regulation. The specificity of Pr1 induction should for the first time make the identification of such an inducing peptide possible. Addition of peptides with sequences similar to the repetitive sequences in locust cuticle (see section 4.1) to established derepressed mycelia might result in high Pr1 levels and be the first identification of an inducer of a microbial protease. Alternatively, peptides released from digesting cuticle with either Pr1 or Pr2 could be separated and effective fractions purified by HPLC and their amino acid sequence determined.

Once the putative Pr1 and Pr2 genomic clones have been confirmed protease induction could be studied at the DNA level. Scanning the DNA sequence would identify any putative regulatory sequences such as pyrimidine-rich regions or TATAA and CAAT motifs upstream of the transcriptional initiation site. Such sequences have been identified in some, but not all, fungal genes.

Both Pr1 and Pr2 are subject to nitrogen catabolite repression, therefore, Metarhizium may possess a gene similar to the nit-2 (N. crassa) and areA (A. nidulans) genes, which are positive trans-acting regulatory genes that activate a number of structural genes involved in nitrogen metabolism. Scanning the Pr1 and Pr2 upstream sequences may identify a "GATA" binding factor sequence which binds the DNA-binding, "zinc- finger" proteins encoded by these genes (eg. Fu and Marzluf, 1990; Jarai et al. 1992). Carbon catabolite repression in A. nidulans has recently been shown to be mediated by a negative regulatory protein encoded by creA (Felenbok et al., 1991). The creA repressor, like the areA protein is also a DNA-binding protein which bind to sequences localised in the 5' regions of genes under the control of carbon catabolite repression. The construction of PR1 gene fusions with either the β-galactosidase (eg. Davis et al., 1988) or β-glucuronidase (eg. Roberts et al., 1989) genes of E. coli could be used to identify and analyse such regulatory sequences. Transforming wild-type M. anisopliae with chimaeric genes containing sequential promoter deletions (eg. Hamer and Timberlake, 1987) would identify sequences responsible for induction and also for carbon and nitrogen repression.

Recent studies have provided strong evidence that Pr1 is a prerequisite for cuticle penetration (reviewed by Charnley and St. Leger, 1991). Briefly, protease is first in a sequence of cuticle degrading enzymes produced in vitro on host cuticle and Pr1 is adapted to extensively degrade cuticular protein (St Leger

et al., 1986b, 1987a), Pr1 is the only cuticle degrading enzyme produced in high amounts by all pathogenic isolates of entomopathogenic fungi (St. Leger et al., 1986b), Pr1 is produced in high levels by the fungus during host penetration (St. Leger et al., 1987c) and specific inhibition of Pr1 delays disease symptoms and mortality of the tobacco hornworm M. sexta (St. Leger et al., 1988a). This information and the unique regulation of Pr1 (ie. induction by a component of cuticular protein) presumably reflects the adaptation of M. anisopliae to insect parasitism.

The biochemical evidence described suggests a key role for Pr1 in pathogenesis and has led to the suggestion that Pr1 is a pathogenicity determinant of M. anisopliae (St. Leger et al., 1988a). Confirmation of this hypothesis has been delayed by the inability to isolate mutants deficient in Pr1 activity using conventional mutagenic techniques, such as UV irradiation and NTG mutagenesis. Once genomic clones of Pr1 have been characterised an alternative approach would be to generate Pr1 mutants by site-directed mutagenesis of Pr1 by gene replacement.

The creation of specific mutations by "reverse genetics" has been used successfully in a number of filamentous fungi, for example A. nidulans (Wernars et al., 1987), Ustilago maydis (Holden et al., 1989) and Cochliobolus carbonum (Scott-Craig et al., 1990). An appropriate internal fragment of the Pr1 cloned into a transformation vector could be used to transform Metarhizium. Any

homologous recombination could result in a loss of Pr1 activity and these mutants could be tested for pathogenicity towards M. sexta larvae or locusts. Transformation of these mutants with the Pr1 gene should restore Pr1 activity and a comparison of the pathogenicity of $Pr1^-$ with mutants restored to $Pr1^+$ would provide critical evidence for the role of Pr1 in cuticle penetration and pathogenesis.

An efficient method for the stable introduction of exogenous DNA is required before the molecular approaches described can be performed. Metarhizium has been transformed to benomyl resistance at the low frequency of 0.1 transformants per μg DNA (Bernier et al., 1989; Goettel et al., 1990). This frequency of transformation would need to be increased before any extensive molecular studies could be performed efficiently. Nitrate reductase mutants of A. niger (Unkles et al., 1989a) and A. oryzae (Unkles et al., 1989b) have been transformed using the homologous nitrate reductase genes. Heterologous transformation of Fusarium oxysporum (Malardier et al., 1989) and seven agriculturally and industrially important species of filamentous fungi, including the entomopathogen B. bassiana (Daboussi et al., 1989) has been reported using the nitrate reductase gene from A. nidulans, with transformation frequencies ranging from 1-10 transformants per μg DNA. It is likely that a similar transformation system could be developed for Metarhizium and might generate a higher transformation frequencies than the benomyl system. As a first step towards the development of such a

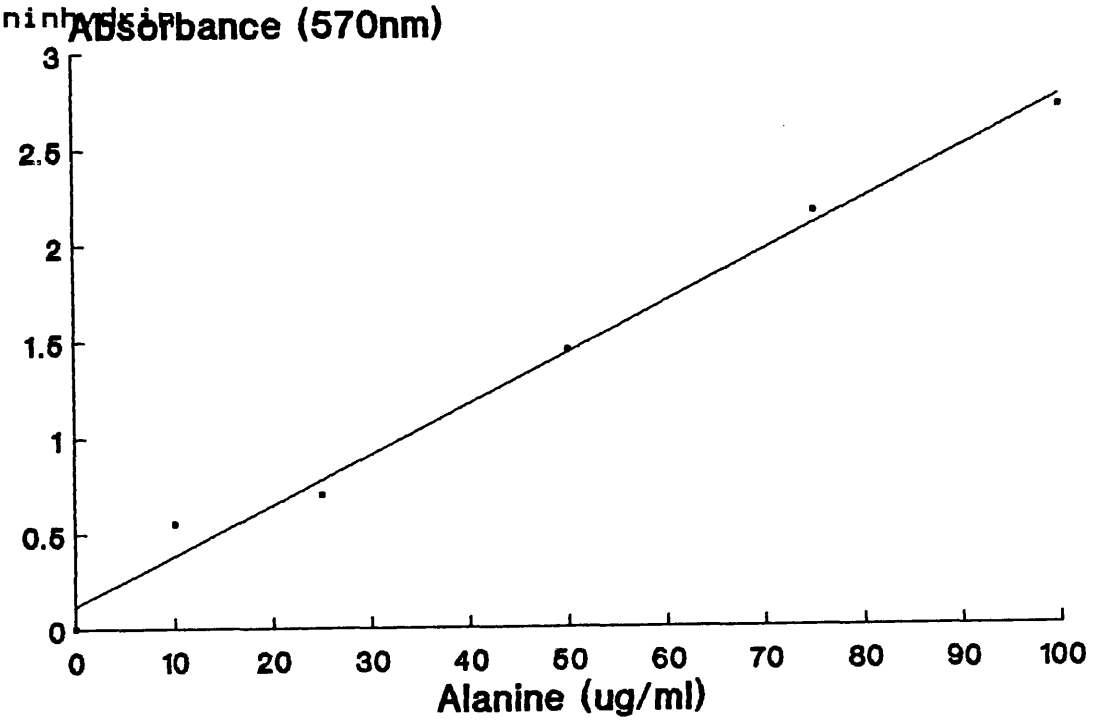
transformation system, the author has identified nitrate reductase mutants of M. anisopliae strain ME1 by selection for chlorate resistance and subsequent characterisation by simple growth tests (Cove, 1979).

Now a transformation system for Metarhizium has been developed and a pathogenicity and/or virulence gene cloned, it is possible, for the first time, to attempt a molecular approach to rational strain improvement for entomopathogenic fungi. Transformation in filamentous fungi frequently occurs with the integration of multiple copies of plasmid sequences into the genome (Fincham, 1989) and it is possible that increasing the copy number of pathogenicity genes in this way might increase their expression (Clarkson, 1992). Single gene products like cuticle-degrading enzymes and some peptide toxins lend themselves to this approach. Combining a number of virulence factors in a single strain by genetic manipulation may lead to accelerated host kill and thereby increase the utilisation of entomopathogenic fungi in the biocontrol of insect pests.

APPENDICES

Appendix 1

Calibration curve of L-alanine assayed for amino groups with ninhydrin



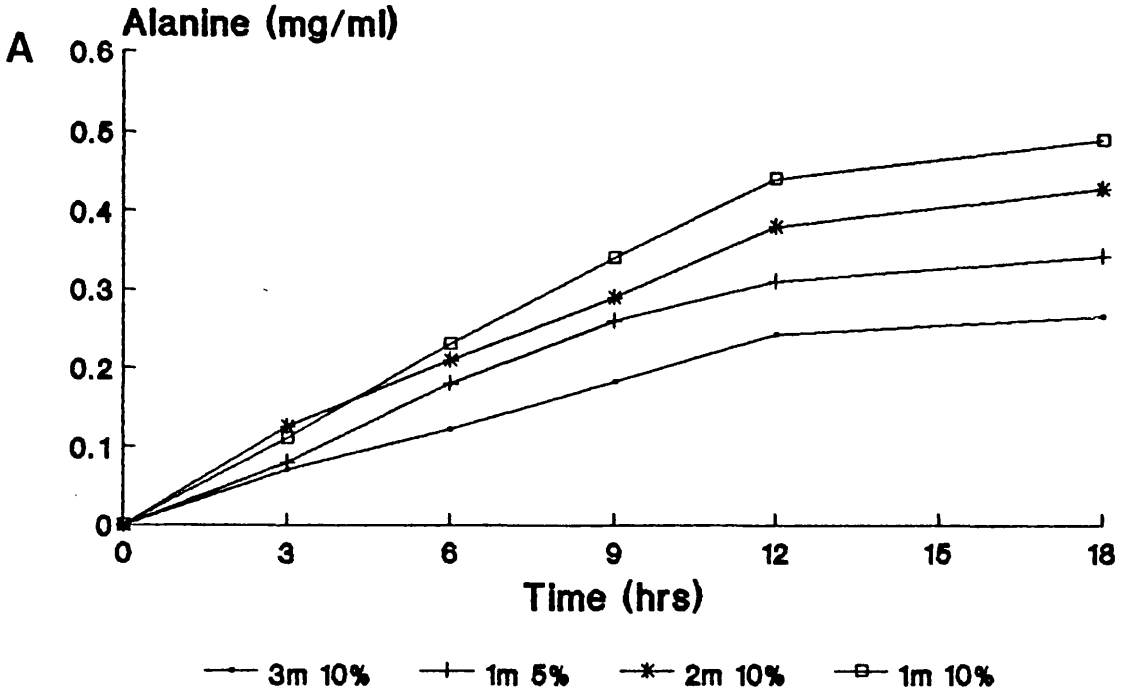
L-alanine at concentrations of 10, 25, 50, 75 and 100 $\mu g\ ml^{-1}$ was assayed with ninhydrin as described in the text. Ninhydrin was measured by absorbance at 570 nm.

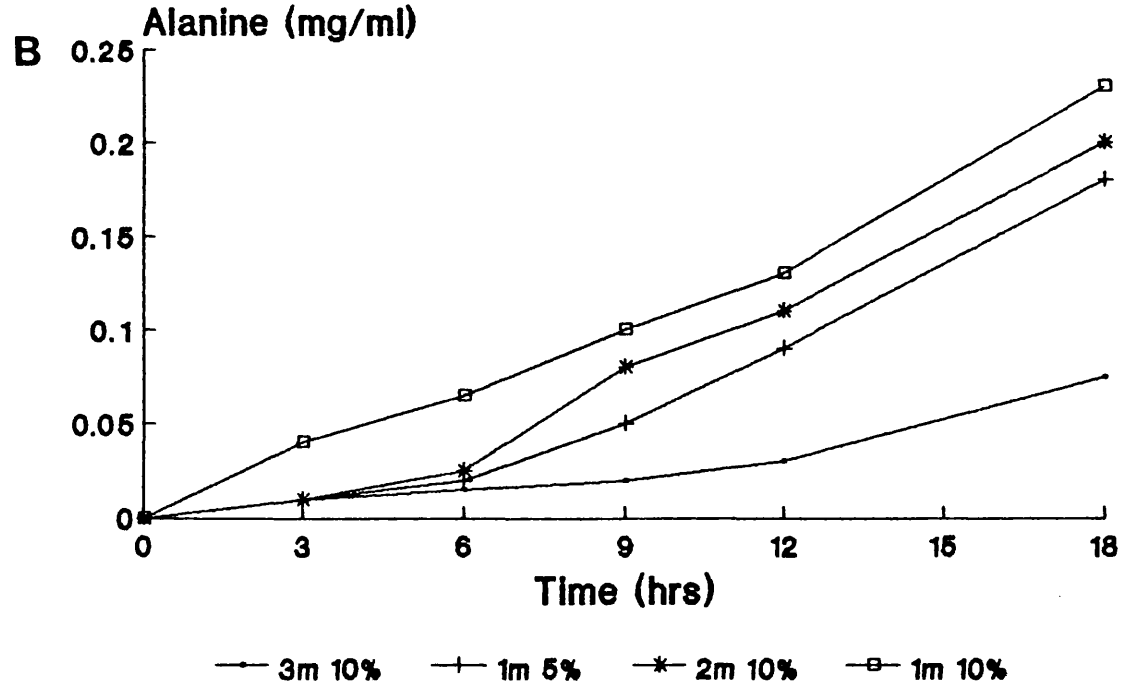
Appendix 2

Diffusion of monomeric and dimeric L-alanine from diffusion capsules

Diffusion capsules containing 5% or 10% (w/v) solutions of either monomeric (A) or dimeric (B) L-alanine were placed in 100ml of buffered basal salts and incubated for 18 hrs at 27°C, 150 rpm. The diffusing amino acid was detected with ninhydrin. Capsules contained either 1, 2 or 3 membranes (dialysis tubing). M = number of membranes.

Appendix 2


A
Alanine (mg/ml)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
3
6
9
12
15
18
Time (hrs)
3m 10%
1m 5%
2m 10%
1m 10%


B
Alanine (mg/ml)
0.25
0.2
0.15
0.1
0.05
0
0
3
6
9
12
15
18
Time (hrs)
3m 10%
1m 5%
2m 10%
1m 10%

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